RECOMBINANT BCG OVEREXPRESSING C-DI-AMP DEMONSTRATES ENHANCED IMMUNOGENICITY AND ENHANCED ANTI-TUMOR CYTOTOXICITY IN MNU-INDUCED BLADDER CANCER

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Abstract

STING-dependent Type I IFN responses have taken on heightened interest with the realization that STING agonists could be an important adjunct therapy to the cell-cycle check point inhibitors being used against several cancers. Intravesical instillation of BCG remains at the heart of bladder cancer immunotherapy to specifically treat the non-muscle invasive forms of this tumor (NMIBC) that accounts for more than 75% of the cases. Besides being an effective immunodominant antigenic vessel, BCG combined with Type I IFN (IFN-α2β) shows remarkable promise against bladder cancer, even in patients' refractory to BCG-only immunotherapy. The non-canonical activator of STING, c-di-AMP, contributes to induction of Type I IFN as well as the activation of pro-inflammatory cytokines from mammalian myeloid cells. Pathogenic prokaryotes have evolved well-established mechanisms of synthesis and release of cyclic di-nucleotides including c-di-AMP that are important for basic physiological regulation as well as in the pathogenesis of infection. *Mycobacterium tuberculosis* encodes a critical enzyme, the cyclase, *disA* (Rv3586) that plays an important role in bacterial pathogenesis. *M. tuberculosis disA* is responsible for c-di-AMP synthesis that is a potent inducer of STING-dependent Type I IFN production by macrophages. We generated a recombinant BCG strain that overexpresses (BCG-*disA* OE) c-di-AMP, induces STING/TBK1/IFN-β. We then tested these strains in rodent model of urinary bladder cancer for their immunotherapeutic potential. Our preliminary results show an improved treatment outcome in the *in vivo* bladder cancer model and predict STING agonist like properties of c-di-AMP overexpressing BCG. Further investigations are needed to identify the direct immunomodulatory role of BCG-*disA* OE at the tumor microenvironment. We do not rule

out the possibility of checkpoint inhibitor and STING agonist like BCG based combination therapy to test the immunotherapeutic potential of BCG against NMIBC in general and BCG-refractive cases, in particular.

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Chapter 1

Introduction

a. Tuberculosis and BCG vaccine

With an estimated 10.4 million incident cases and 1.7 million deaths reported in 2016, tuberculosis (TB) remains one of the top infectious killers of humanity. The resurgence of TB as a global epidemic has been triggered by the HIV/AIDS pandemic, and the emergence of drug-resistant strains of *Mycobacterium tuberculosis* (*M.tb*), among other factors (WHO global tuberculosis, 2017).

TB is the leading cause of mortality in HIV-positive individuals [1]. For the last 90 years, Bacillus Calmette-Guérin (BCG), an attenuated strain of *Mycobacterium bovis*, remains the only available vaccine against TB, and it is widely administered to infants in areas endemic to TB. The vaccine has been effective in the prevention of extra-pulmonary TB in children, such as tuberculous meningitis and miliary TB [2], but has variable efficacy in adults and adolescents, ranging from 0-80% [3]. This variation has been attributed to human genetic variations, pre-exposure to endemic mycobacteria, and to geographical differences, with poor efficacy in high burden regions [3]. The insufficient protection conferred by BCG against pulmonary TB is believed be one of the major reasons for rapid spread of the disease, and therefore global efforts to improve its efficacy and to develop newer vaccines have intensified.

During initial infection, *M.tb* is engulfed by alveolar and interstitial lung macrophages, as well as lung resident dendritic cells (DCs). In these cells, *M.tb* is able to arrest the conventional phagosome maturation process, and makes the intraphagosomal compartment amicable for bacterial survival and their replication [4-7]. Moreover, *M.tb* and *M.bovis* are known to release cell wall lipids and effectors that can modulate host responses [4,8-11].

Host adaptive immunity is triggered by the processing of mycobacterial antigens by DCs that activate T lymphocytes, and is further enhanced by pro and anti-inflammatory cytokines released during infection, which promote myeloid and lymphocyte cell recruitment. Type I IFN (IFNα/β) released during *M.tb* infection affect downstream signaling pathways and several transcription factors such as NF-κB, leading to induction of a large number of IFN-stimulated genes (ISGs) and production of IL-1β and other key pro-inflammatory mediators such as TNF, and IL-6, all of which stimulate vigorous anti-microbial responses [12].

BCG is also believed to mediate immunity through the development of antigen-specific memory T cells [2,13], which protect against disseminated TB and TB meningitis, but the vaccine is inefficient against pulmonary TB in all individuals for reasons that are not well understood. This has led to ongoing efforts to modify and improve BCG, and a number of candidate vaccines are currently being evaluated in clinical trials [14]. Efforts to improve BCG have focused on the overexpression of immuno-dominant antigens of BCG or on the reintroduction of any beneficial antigens that may have been lost during BCG attenuation [15].

b. BCG as immunotherapeutic in diseases other than TB

Ever since the introduction of BCG as a vaccine for TB, clinicians have reported the heterologous effects of BCG on different unrelated diseases ranging from viral infections to various cancers.

In 2002, Ota et al. first highlighted that patients that received BCG priming prior to other neonatal vaccinations such as hepatitis B vaccine, tetanus toxoid vaccine and oral polio vaccine produced a higher antibody titer as compared to the controls who did not receive priming with BCG [16]. Other studies corroborated the induction of superior immune responses to nontuberculous pathogens after BCG priming [17-20]. One conscientious study by Mathurin et al. in 2009 delineated the heterologous immunity conferred in an *in vivo* mouse model in the BCG immune mice to Vaccinia virus infection. This study also addressed that the underlying immunity conferred by BCG vaccination to the viral infection is due to increased induction of CD4 T cells [21].

Some pioneer studies indicate application of BCG as anticancer therapy and gave insights into usage of BCG in cancers. Holmgren, in 1936, reported application of BCG as a therapeutic intervention for stomach cancer in patients [61]. Subsequently, in 1959, Old et al., for the first time showed that intravenous injection with BCG prior to transplantable sarcoma tumor challenge in mice resulted in retardation of tumor growth and increased the survival [22]. In another study, investigators measured the immunological reaction induced, against different types of tumor isografts after intraperitoneal injections of BCG, in a mouse model and reported a superior anti-tumor immune response [23].

After proving that BCG priming resulted in the retardation tumor growth and enhanced immune reactions, the Zbar and Tanaka group set out to study effect of vaccination for longer durations in a guinea pig model for with hepatocarcinoma. In addition to tumor regression, a notable outcome fact was that the BCG-treated guinea pigs were refractory to tumor development, which brought focus on the necessity of pre-existing immunity on the outcomes of BCG treatment [24]. In patients suffering with a wide array of gastric cancers (stomach, colon to pancreatic cancer), the utility and efficacy of BCG as an adjuvant to chemotherapy was tested for by Falk and his group. Intraperitoneally administration of BCG to patients followed by several cycles of oral BCG achieved prolonged survival in most patients [25]. Different groups suggested usage of BCG against melanoma after encouraging results in cancers such as adenocarcinomas and sarcoma were reported [64].

In a clinical study Morton et al., reported that intralesionally injected BCG in a cohort of 36 subcutaneous melanoma patients led to complete lesion regression in 684 out of 754 lesions [26]. In a similar clinical study, Rosenberg and Rapp reported that recurrent cutaneous melanoma nodules disappear 90% of the time when injected with BCG at the tumor site [27].

BCG as an immunotherapeutic was also tested in urinary cancers like prostate cancer in 1982. In a clinical study done by Guinan et al., intratumoral injection of BCG in addition to conventional chemotherapy showed prolonged survival of the patients [28]. In all, BCG treatment as an immunotherapeutic in both *in-vivo* and clinical studies has been successful in showing tumor regression and heightened enhanced immune response.

c. BCG in Bladder cancer

Bladder cancer consists of an array of diseases with wide pathologies and prognoses. Majority (~90%) are known to be urothelial carcinomas that primarily begin with transitional epithelial cells (also known as transitional cell carcinoma). The remaining 10% are classified as squamous cell carcinoma (which begin with flat squamous cells forming the bladder), small cell carcinoma (start with neuroendocrine cells of the bladder), or adenocarcinoma (which primarily starts with the glandular cells in the bladder) [144]. Approximately 75% of patients with bladder cancer present with a disease that is confined to the mucosa or submucosa, grouped as non-muscle invasive bladder cancer (NMIBC) [145]. They are moreover classified into low grade i.e. papillary (which do not invade the muscle layer) or they can progress to a high grade which are superficial tumors as well as carcinoma *in situ* (a flattened layer of dysplastic cells on the surface of bladder that can progress into muscle invasive forms of cancer) [79]. NMIBC is characterized as carcinoma in situ (CIS), Ta (low grade noninvasive) and T1 (muscle invasive into lamina propria) stages. Muscle invasive form of the disease only accounts for less than 25% of the cases and has a 5-year survival rate in only 50% cases. Further if the tumor metastasizes then the 5-year survival rate is only 15% [79, 145].

Over the last 40 years, BCG immunotherapy remains the gold standard to treat NMIBC. Till date, it represents the only agent known to reduce the recurrence and the progression into muscle invasive bladder cancer [29]. BCG immunotherapy involves transurethral resection of the tumor followed by intravesical instillation of BCG. It shows superiority to standard transurethral resection (TUR) with any chemotherapeutic agents like mitomycin C. BCG as an intravesical immunotherapeutic for NMIBC was pioneered

by Bloomberg and group [30]. They sensitized mongrel dogs with purified protein derivative of tuberculin, and measured the effects intravesical instillation of BCG into the bladders of sensitized or unsensitized dogs. They found that BCG elicited an inflammatory reaction, particularly in sensitized dogs, which seemed to be temporary and well-tolerated by subjects [30]. This pioneering study paved to the first important approach in 1976 used by Morales et al. to administer intravesical instillation of BCG following transurethral resection in 9 patients which significantly reduced the recurrence of bladder cancer [31]. Given the enormous number of persons currently vaccinated with BCG worldwide (more than 3 billion), understanding how BCG influences the host response to these wide arrays of diseases from infectious diseases to cancer is an important issue for clinical research and practice. Also, typical complete response rates for BCG treatment are 55-65% for papillary tumors and 70-75% for carcinoma *in situ* (CIS). The burden of patients with BCG unresponsive and relapsing disease and of those intolerant of treatment has therefore prompted the need for further improving the efficacy of BCG against NMIBC.

d. Synthetic cyclic dinucleotide (CDN) STING Agoinsts in Cancer Immunotherapy

In human melanomas, Gajewski et al. in 2007, employed analysis of gene expression profiles to reveal that Type I Interferon (IFN) is a key marker for tumors that are highly infiltrated with CD8+ T cells indicating for the first time that Type I IFN might be the important bridge between innate and adaptive immunity [32].

In 2011, studies in animal models further elucidated the roles that Type I IFN signaling has in host cells and in the augmentation of a spontaneous anti-tumor $CD8^+$ T cells [33-34]. It

is now well established that Type I IFNs, produced both by innate immune cells in tumor microenvironment and by the tumor cells themselves, are known to mediate anti-tumor effects against several malignancies [33-36].

It wasn't until 2014, when Woo et al. showed that tumor DNA activated cytosolic STING pathway by triggering the production of IFNβ in dendritic cells, that the role of STING came into focus. Their work indicated that in STING deficient hosts, localized tumors, were not capable of inducing a spontaneous $CD8^+$ T cell responses, which was not observed when the mice were deficient in other innate signaling pathways [35].

The prominence of STING as a target for development of immunotherapy further came into light when Juan Fu and group showed that cellular cancer vaccines formulated by CDN ligands demonstrated *in vivo* antitumor efficacy via potent induction of STING. They also made a remarkable observation that made was this potent induction of STING led to an upregulation of PD-1. When they combined CDN therapy with a PD-1 blockade therapy, they observed tumor regression of otherwise anti-PD-1 resistant tumors [36].

Many groups have tried CDNs as adjuvants for activation of STING in the tumor microenvironment to mediating anti-tumor immunity in acute myeloid leukemia, chronic lymphocytic leukemia, gliomas and head and neck squamous cell carcinoma [37-40].

The compound 5,6-dimethylxanthenone-4-acetic acid (DMXAA), a flavonoid compound known for vascular disruption showed anti-tumor activity in mouse models, has been used in 1997 by Ching et al. [41] for its promising anti-cancer properties. However, DMXAA failed in a phase 3 efficacy trial in non-small cell lung cancer [42].

Subsequent studies showed that DMXAA only bound to murine STING and not human STING. This was due to the polymorphisms in the mouse and human STING proteins. These findings provided a mechanistic insight into for the lack of DMXAA efficacy in humans, as well as the rationale for the development of new pharmacologic compounds that potently activate human STING sensing system [42].

Corrales et al. studied other synthetic STING agonists and used them for intratumoral injections into established tumors in melanoma, colon and breast carcinoma models and observed marked tumor regression and systemic T cell immunity that was tumor antigen specific. Further they sorted mouse bone marrow derived macrophages, DCs, lymphocytes and endothelial cells after *ex vivo* stimulation with the other synthetic CDNs. They noted that the highest IFNβ was produced by macrophages followed by DCs, and to lesser extent in lymphocytes and endothelial cells [47-48].

A recent study by Francica et al., using knockout chimeric mouse model was able to highlight the other pleiotropic effects of STING activation on induction of TNF, which is postulated to be necessary for CDN induced necrosis. Moreover, they also illustrated that in addition to myeloid cells, signaling by STING is important for the role of stromal cells for tumor rejection [49].

The cancer-specific STING agonists being developed by various groups to be used as an adjunct therapy are outlined in **Table 1**.

Table 1. STING Agonists under different developmental stages		
Aduro Biotech (Berkeley, CA)	ADU-S100, a synthetic cyclic dinucleotide	Phase I clinical trials in solid tumors and lymphomas: monotherapy or in combination with PD-1 blockade (Novartis)
Bristol-Myers Squibb	Small-molecule pathway modulator(s)	Preclinical; portfolio acquired from IFM Therapeutics (Boston, MA) in August 2017
Curadev (India)	Small-molecule pathway modulator(s)	Preclinical
iTeos Therapeutics (Belgium)	Small-molecule pathway modulator(s)	Preclinical; partnering with Cristal Therapeutics (Netherlands) on nanoparticle-based targeted delivery strategy
Mavupharma (Kirkland, WA)	Small-molecule conditional pathway modulators; orally bioavailable	Preclinical; completed a $20 million Series A financing in December 2017
Merck	MK-1454, a synthetic cyclic dinucleotide	Phase I clinical trials in solid tumors and lymphomas: monotherapy or in combination with pembrolizumab (Keytruda)
Nimbus Therapeutics (Cambridge, MA)	Small-molecule pathway modulator(s)	Preclinical
Selvita (Poland)	Small-molecule pathway modulator(s)	Preclinical
Spring Bank Pharmaceuticals (Hopkinton, MA)	SB 11285, designed for intravenous delivery as an antibody–drug conjugate	Aims to start phase Ib/II trials in liver cancer in 2018

e. Genetically modified BCG for cancer immunotherapy

Numerous independent research groups have attempted to express mammalian cytokines to enhance the immunotherapeutic efficacy of BCG in bladder cancer. From early on in bladder cancer therapy, a higher Th1 cytokine profile characterized by IL-2, IL-12 and IFNγ in patient samples has been shown to be favorably associated with BCG response and tumor regression. Many groups have hence tried to express these cytokines using BCG as a delivery vehicle for better immunotherapy in urothelial cancers [50-56].

For example, in one of the earliest studies done in a SCID mouse model with MUC-1 positive tumors by Chung et al., showed higher growth inhibition using rBCG expressing tumor antigen MUC-1 plus human IL-2 (BCG-hIL2-MUC1) [57].

Further details of other trials that have constructed rBCG and the type of immunity expressed is outlined in the **Table 2**.

Table 2. Developed rBCG strains used against Bladder cancer

Recombinant Strain	**Model System used**	**Treatment outcomes**
rBCG-IL18	*In vivo* mouse model and *in vitro* BC cell line (MBT-2, PEC)	Macrophage cytotoxicity in vitro, IFN-γ secreting cell proliferation
rBCG-IL2	*In vitro* BC cell lines (PEC and MBT-2)	Macrophage cytotoxicity against MBT-2, IFNγ and IL-12 induction
rBCG-IFN-α-2β (Pasteur strain)	*In vitro* (PBMC and BC cell lines (T24, J82, 5637, UMUC-3, TCCSUP))	PBMC cytotoxic against BC cell lines, significantly increased IFNγ, IP-10 and IL-2
rBCG-IFN-α-2β (Danish strain)	*In vitro* (PBMC and BC cell lines (T24 and T5637)	PBMC cytotoxicity against BC cell lines
rBCG-IFNγ	Mice and *in vitro* (MB49 BC cells and splenocytes)	Increased MHC-I induction *in vitro* in BC cells, Recruitment of $CD4^+$ T cells *in vivo*, Increased IL-2 *in vivo*, and Increased survival of mice
rBCG-IL2-IL18	*In vitro* (PEC and MBT-2 cell line)	Increased IFNγ, TNF-α and IL-6
rBCG-Pertussis toxin (SIPT)	*In vivo* Mice orthotoptic tumor model	Increased TNF-α and IL-10 induction
rBCG-IFN- α-2β	*In vitro* (PBMCs and BC cell lines)	Cytotoxicity of PBMC against BC cell lines and increased proliferation of PBMCs
rBCG-IFN-α	*In vitro*	Antitumor effects and Th1 cytokines induction, superior response and effect when compared with clinical combination therapy of BCG+IFN-α-2β

Detailed discussion of further rBCG in bladder cancer and responses elicited will be mentioned in the following chapters. The most potent adjunct therapy so far has been Type I IFN (IFN α-2b) in conjugation with BCG in preclinical and further clinical trials. Recent work in STING-mediated Type I IFN induction in other cancers, paves way for possible mechanistic modifications.

f. BCG encoding CDNs: STING agonist like BCG

c-di-AMP, produced primarily in gram positive bacteria plays myriad roles in bacterial growth, regulation of virulence and even survival during adverse stress conditions. This activity and level of c-di-AMP at the cellular level is primarily due to two key enzymes-c-di-AMP synthesizing enzymes, diadenylate cyclases (DACs), and degradation enzymes, phosphodiesterases (PDEs) [58]. The importance of *disA/dacA* gene in the production of adenylate cyclase and subsequent circularization of ATP or ADP to form c-di-AMP has been well characterized in many gram-positive bacteria like in *S. aureus* [59], *L. monocytogenes*[60], *M. tuberculosis* [61] and *B. subtilis* [62]. Genetic manipulations of these genes have shown a prominent role of c-di-AMP in genotoxic stress mediated DNA damage, homeostasis, growth of the bacteria and virulence of microorganisms in the host [63].

Small molecules like cyclic di-nucleotides (CDNs) are a class of molecules that are known to activate host cytosolic surveillance pathways (CSP) in eukaryotic cells through an array of host proteins. CDNs are produced endogenously to response of foreign DNA or secreted by invading pathogens. Detection of c-di-AMP, a pathogen associated molecular pattern (PAMP) by the host CSP induces Type I IFNs, a family of pro inflammatory cytokines [64-

65]. The likelihood of applicability of CDNs and structurally related compounds as danger signal (for adjuvant therapy) was tested when c-di-IMP (synthesized from c-di-AMP through adenosine deamination), induced a strong *in vivo* as well as *in vivo* immune responses [66-67]. Other intracellular bacteria such as *M. tuberculosis* [61], Group B Streptococcus [68] and *Chlamydia trachomatis* [69] were found to secrete CDNs that activated the mammalian cGAS/STING axis.

Another prominent mechanism of STING activation is the detection of dsDNA released into the cytosol. Cytosolic double stranded DNA (dsDNA) binds to enzymatic protein cyclic GMP-AMP (cGAMP) synthase (cGAS), that converts ATP and GTP into cGAMP [70]. As a damage associated molecular pattern (DAMP) molecule, cGAMP binds to stimulator of interferon genes (STING/TMEM173), an endoplasmic reticulum resident membrane protein that, after binding of cGAMP, phosphorylates adaptor protein TANK-binding kinase 1 (TBK1). TBK1 then phosphorylates interferon response factor 3 (IRF3), a transcription factor that then translocates into the nucleus and mediates the transcription of multiple interferon-stimulated genes (ISGs), including IFNα/β and other co-regulated host defense pathways [71]. Both bacterial as well viral pathogens are known to exploit cGAS/CDN/STING pathways, for example intracellular bacterium *Listeria monocytogenes* produces cyclic-di-adenosine monophosphate (c-di-AMP) that binds to STING and activates IFNβ and other co-regulated genes [60].

Host cells recognize and respond to dsDNA viruses, such as pox viruses, adenoviruses, gamma herpes viruses and retroviruses, by synthesizing cGAMP, that then gets transported to adjacent cells via gap junctions and hence activate IFNα/β synthesis in neighboring cells thus preventing viral dissemination [72-76].

Previous findings from the Bishai Laboratory showed that *M.tb* synthesizes and secretes c-di-AMP, which activates the IRF pathway and Type I IFN responses through cGAS/STING. Importantly, c-di-AMP overexpressing *M.tb* strains show attenuation of TB in a mouse model [77].

The remarkable ability of bacterial CDNs and host induced cGAMP to activate cGAS/STING axis in immune cells make them a new class of immune modulators, called the 'STING agonists' that specifically activate Type I IFN synthesis in TLR-independent fashion.

In the thesis work presented here, we developed BCG overexpressing STING agonist c-di-AMP to test induction of STING-dependent Type I IFN and proinflammatory cytokines *in vivo* using a rodent model of urinary bladder cancer. Evidences from our *in vivo* experiments show improved immunotherapeutic potential of BCG-*disA* OE (Recombinant BCG harboring and overexpressing *disA* gene) over the conventional BCG in therapy, against bladder cancer further complementing the new era of BCG engineering to generate the next generation of immunotherapeutic tools against bladder cancer.

Chapter 2

Materials and Methods

1. Bacterial strains and culture conditions:

In this study we have used *Mycobacterium bovis* (*M. bovis*) Bacillus Calmette-Guérin (BCG) Pasteur strain and *Mycobacterium tuberculosis (M.tb)* strain CDC1551. The BCG Pasteur strain was a kind gift from Frank Colins from FDA. Bacterial strains were stored in frozen vials at -80°C, and revived and subsequently sub-cultured in 7H9 Middlebrook liquid medium (Cat. B271310, Fisher Scientific) supplemented with oleic acid-albumin-dextrose-catalase (OADC) (Cat. B11886, Fisher Scientific), 0.5% glycerol (Cat. G5516, Sigma) and 0.05% Tween-80 (Cat. BP338, Fisher Scientific). For gene cloning and protein expression studies *E. coli* strains DH5a (18258012, Fischer Scientific) and BL21DE3 (C2527I, New England *Biolabs* Inc) strains were routinely used. A detailed description of different bacterial strains, plasmids and oligos presented in **Table 4**.

2. Preparation of frozen stocks and competent cell preparations:

Fresh frozen vials for *M.tb* and BCG strains were prepared using 10^8 bacilli (OD_{600}:1) in 7H9 Middlebrook media in 50% sterile glycerol and immediately stored at -80°C as described earlier [61]. For infection and instillation purposes fresh vials were revived in 7H9 media and were grown for 5 subculture cycles to avoid variations. For competent cell preparations, standard protocols were followed as described elsewhere. Briefly, log phase (OD_{600}:0.6-0.8) mycobacterial cultures were pelleted and repeatedly washed using 10% sterile glycerol solution and were finally stored in 10% glycerol in 300 µL (approximately

10^{11} cells/mL) volumes and stored at -80°C. For electroporation 100 µL competent (approximately 10^{10} cells) cells were used per 2-4 µg of plasmid.

3. Genomic DNA isolation from *M.tb*-CDC 1551:

Genomic DNA isolation was performed using CTAB method from log phase cultures of *M.tb*-CDC 1551 as described previously. The quantity and quality of high molecular weight genomic DNA was determined using NanoDrop and DNA agarose gel analyses, respectively.

4. Cloning *M. tuberculosis disA* (MT3692) into mycobacterial expression vector pSD5-hsp60:

Using gene-specific primers, pSD5hsp60.MT3692 (F) and pSD5hsp60.MT3692 (R), the *disA* (MT3692) gene of *M.tb*, (**Table 3**) was PCR amplified using high molecular weight genomic DNA isolated from *M.tb* CDC-1551 as the template. PCR conditions were 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 1 minute and a final extension step at 72°C for 10 minutes. Gene amplicons were cloned into mycobacterial shuttle vector pSD5-hsp60 at the NdeI and MluI restriction sites. The clone (pSD5-hsp60-MT3692) was confirmed by insert release and sequence analyses.

5. Overexpression of *disA* (MT3692) in BCG Pasteur:

The construct pSD5-hsp60-MT3692 was subsequently used to transform BCG using electroporation method [78]. Briefly, 2-4 µg of clone (pSD5-hsp60-MT3692) was added to 100 µL of electrocompetent cells and mixed gently by pipetting several times. The mix was transferred to pre-chilled electroporation cuvettes. Moisture was removed before

inserting the cuvette into the device. For electroporation, the following conditions were used: voltage (V) 2,500, 25μF and resistance 1,000Ω as per set protocol [78]. Immediately, 1ml of 7H9 Middlebrook media devoid of antibiotic was added to cells and bacilli were incubated at 37°C for next 48hours with agitation. Between 50 and 100 μL of cells were subsequently plated on 7H11 selection plates with kanamycin (25 μg/mL). Single colonies were picked and were inoculated into 7H9 Middlebrook liquid medium with kanamycin (25 μg/mL) for subsequent propagation. Recombinant clones were confirmed using colony PCR against kanamycin cassette (**Table 3**). MT3692 overexpression phenotype of BCG-*disA* OE strain was further confirmed by mRNA expression using quantitative real time PCR (qPCR).

6. Colony PCR:

For colony PCR, bacterial colonies were picked using sterile tips and were inoculated in 50 μL of 0.1% Triton X100 made in nuclease free water and the mixture was boiled for 5 minutes at 100°C, followed by snap chilling. Mixture was centrifuged and supernatant was filtered using spin-column before performing gene-specific PCR. Small volume (10 μL) of sterile supernatant was used to perform PCR against kanamycin cassette specific primers (**Table 3**). Genomic DNA isolated from wild-type BCG and pSD5.hsp60 shuttle vector were used as controls.

7. Quantitative real-time PCR (qPCR) for *M.tb disA*:

Late log phase BCG cultures were harvested and bead beating was carried out using zirconium beads (Cat. KT03961-1-102.BK, Berlin Technologies) before performing total RNA isolation using Trizol method (Cat. 15596026, Fischer Scientific) as suggested by

manufacturer. The quality and quantity of RNA was determined using DNA gel analyses and Nanodrop. Quantification of mRNA, amplification, and quantification of cDNA was carried out using SYBR Fast green double stranded DNA binding dye (Cat. 4085612, Applied Biosystems, USA) and ABI StepOnePlus Real Time PCR System (Applied Biosystems, USA). Amplification of *M.tb sigH* (**Table 3**) was carried out as an internal control. PCR was performed at 95 °C, 2 minutes, followed by 40 cycles of 95 °C, 5 seconds; 60 °C, 30 seconds. Melt curve analyses confirmed formation of desired and specific PCR product. Experiments were performed in triplicate using three independent biological samples and results were analyzed and presented using $2^{-\Delta\Delta CT}$ method (25730264). Details of NCBI gene identifiers and primer sequences are mentioned (**Table 3**).

8. Mammalian Cell Culture:

THP-1, an acute monocytic leukemia-derived human cell line (ATCC, TIB-202TM) was cultivated in suspension at a density 10^5 to 10^6 cells/mL in RPMI-Glutamax (Cat. 61870-036, Fischer Scientific) and supplemented with 10% heat inactivated fetal bovine serum (Cat. 10082147, Fischer Scientific) with 1% streptomycin/penicillin at 37°C with 5% CO_2. For differentiation, cells were treated with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma-Aldrich, P1585) for 48 hours to induce differentiation into macrophages. For activation, THP-1 cells were treated with 100U/ml human recombinant interferon gamma (Cat. 300-02, Peprotech) for 6 hours.

J774A.1 (ATCC® TIB67™) murine macrophage cell line was cultivated as monolayer in RPMI-Glutamax (Cat. 61870-036, Fischer Scientific) supplemented with 10% heat inactivated fetal bovine serum (Cat. 10082-147, Fischer Scientific) with 1% streptomycin/penicillin (Cat. 15140-122, Fischer Scientific) at 37°C with 5% CO_2. For

infection assays cells were seeded at desired density for 3 hours for attachment on surface. For activation of J774A.1 cells were treated with 100U/ml mouse recombinant interferon gamma (315-05, Peprotech) for 6 hours.

Mouse bone marrow derived macrophages (Mouse BMDM):

Murine bone marrow was isolated from 4-6 weeks old female C57BL/6J (Charles river). Approximately 10^8 cells were stored in cryomedia (10% DMSO in heat inactivated FBS) overnight at -80°C followed by transfer to deep cryopreservation in liquid nitrogen. For differentiation of bone marrow cells into primary macrophages, bone marrow cells were differentiated for 7 days in presence of RPMI-Glutamax supplemented with 10% heat inactivated fetal bovine serum and antibiotics (Penicillin-Streptomycin solution) and 30% (vol/vol) L929 conditioned media. Mouse fibroblast L929 cells (ATCC® CCL-1™) were maintained in RPMI-1640 medium supplemented with 10% FBS and antibiotics.

Human and Rat bladder cell lines:

Human epithelial grade II carcinoma from bladder tissue 5637 was (ATCC® HTB-9™), Human transitional cell papilloma RT4 (ATCC ® HTB-2™) and N-butyl-N-(4-hydroxybutyl) nitrosamine induced tumor cell line in *Rattus norvegicus* Nara Bladder Tumor No. 2 (ATCC® CRL-1655™) were maintained in RPMI-1640 medium supplemented with 10% FBS and antibiotics was cultured and used as monolayer at 37°C and 5% CO_2. For infection assays cells were seeded at desired density for 3 hours for attachment on surface.

9. *In vitro* infection assays:

For infection assays, macrophages were seeded at desired cell density as mentioned earlier [61]. Briefly, log phase bacterial cultures were repeatedly washed with phosphate buffered saline (PBS) (Cat. 14190144, Life Technologies, USA) to remove any trace of detergents, infection was carried out at 1:20 multiplicity of infection (M.O.I.). Infection was allowed in macrophage infection media (RPMI-1640 with 10% FBS) for 5 hours. Non-infective extracellular bacilli were removed by repeated washing using PBS. This time point was considered 0 hours, and cells were subsequently incubated for different time points till the end-point was achieved.

10. Quantitative real time PCR for Human, Mouse and Rat genes:

Gene expression profiling in human (*Homo sapiens*), mouse (*Mus musculus*) and rats (*Rattus norvegicus*) were carried using different RNA samples originating from primary macrophages (mouse BMDMs), macrophage cell lines (THP-1 and J774.1), and different bladder cancer cell lines (RT4, 5637 and NBT-II) after infection assay or drug treatment. Briefly, Trizol reagent (Cat no. 15596026, Thermo Fischer Scientific) was used for RNA isolation after cells were harvested and quantification of mRNA, amplification, and quantification of cDNA was carried out using SYBR Fast green double stranded DNA binding dye (Cat. 4085612, Applied Biosystems, USA) and ABI StepOnePlus Real Time PCR System (Applied Biosystems, USA) as mentioned earlier (25730264). Amplification of RNU6A (for Human genes), and β-actin (for Mouse and Rat genes) was used as internal control. Experiments were performed in triplicate using three independent biological samples and results were analyzed and presented using $2^{-\Delta\Delta CT}$ method [61]. Details of NCBI gene identifiers and primer sequences are given in the **Table 3**.

11. Measurement of IRF activation:

RAW-Blue ISG (InvivoGen) reporter cells were derived from the murine RAW 264.7 macrophage cell line by stably integrating an interferon regulatory factor (IRF)-inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct (QUANTI-Blue™, Invivogen). The cells were infected with wild-type and *disA* OE strains with a pre-calibrated MOIs for 6 hours. Non-infective, extracellular bacilli were removed by repeated washing using PBS. Cells were incubated for another 18 hours in fresh macrophage infection media (DMEM without antibiotics), and culture supernatants were harvested for determination of IRF activation by a SEAP colorimetric assay using QUANTI-Blue reagent.

12. Cytokine profile using ELISA:

Following *in vitro* infection, culture supernatants were used to perform cytokine ELISA. Briefly, mouse DuoSet ELISA kits (R and D systems, USA) for TNF (Cat. DY410), IL-6 (Cat. DY406-05) and IL-1β (Cat no. 401). Human DuoSet ELISA kits (R and D systems, USA) for TNF (Cat. DY410), IL-6 (Cat. DY206-05), IL-1β (Cat. DY201-05) and MCP-1(DY279-05) were used to quantify the cytokines. The absolute concentrations were determined by referring to a standard curve and expressed as pg/mL as described earlier [61]. For quantification experiments were performed in triplicates.

13. *In vivo* Rat model of bladder cancer:

We have used an immune competent Fisher rat model of bladder cancer that was established at Department of Urology, Johns Hopkins Hospital in collaboration with the Bivalacqua laboratory. This is a proven animal model of bladder cancer and has been used

to test the therapeutic outcomes on bladder tumor after instillation with BCG [79]. For *In vivo instillation of* wild-type and BCG-*disA* OE Pasteur strains, all protocols involving animals followed US National Institutes of Health guidelines and were approved by the animal and care use committee of the Johns Hopkins Medical Institutions as discussed elsewhere [79].

MNU instillation and tumor induction:

For tumor induction, 7- week old Fischer 344 females (avg. weight 160g) were placed with free access to food and water in 12 hours light/dark lighting cycle. N-Nitroso-N-methylurea (MNU), an alkylating agent that is a potent mutagen and cancer-causing agent was directly deposited into the urinary bladder of the rats for tumor induction. Briefly, 3% isoflurane was used for anesthesia, 20G angiocatheter (BD) was placed into the urethra and 300 µl of MNU (1.5 mg/kg) was instilled in the bladder as discussed earlier. A total of 4 instillations of MNU was given to each rat as described earlier and animals were monitored using serial bladder ultrasound at 8 weeks following MNU instillation using the 2100 Visual sonic ultrasound system (Toronto, Ontario, Canada). In order to access the immunological correlates, the immune profile of the MNU model itself, representative animals were sacrificed at 8 and 16 weeks after MNU instillation [79].

BCG instillation and Tissue Harvest: For testing the effect of BCG instillation on bladder tumor, rats were given intravesical administration of 10^8 CFU in a total volume of 300 µl via a 20G angiocatheter per week for a total of six dosages as described earlier [79]. Treatment groups included wild-type and *disA* OE strains of BCG Pasteur. Animals were sacrificed at week 16, bladders were collected for histopathological analyses (n = 5/group),

and areas of cancerous epithelium urothelium were snap frozen for measurement of cytokines and chemokines as described earlier.

14. Immune profiling of BCG treated Rat Bladders:

For gene expression profiling of immunological effectors, areas of cancerous bladder urothelium were identified, dissected, excised and frozen for RNA isolation. Control bladder from normal rat and MNU instilled control rats were also taken out. RNA isolation was performed using Trizol method. Quantification of mRNA, amplification, and quantification of cDNA was carried out using SYBR Fast green double stranded DNA binding dye (Cat. 4085612, Applied Biosystems, USA) and ABI StepOnePlus Real Time PCR System (Applied Biosystems, USA) as mentioned earlier [61]. Amplification of β-actin was used as internal control. Experiments were performed using 5 different biological samples (n=5) and results were analyzed and presented using $2^{-\Delta\Delta CT}$ method. Details of NCBI gene identifiers, primer sequences and PCR product sizes are given in the **Table 3**.

15. Histologic analyses:

For histological analyses, whole bladders were formalin fixed and paraffin embedded. Sections were prepared and stained using hematoxylin-eosin staining as described earlier [79]. A board certified genitourinary pathologist performed the tumor staging in a blinded manner as mentioned previously [79]. Specimens were placed into bins based on the percentage of involvement of abnormal tissue (1=10% involvement, 2=20% involvement and so forth).

16. Statistical analyses:

Fold-expression (qRT-PCR and ELISA) was represented as the mean of 3 independent experiments ± standard error mean (SEM). Student's T test (*P < 0.05 **P < 0.01, ***P < 0.001). MOI (multiplicity of infection).

Table 3. List of primer sequences used for gene expression analysis

Accession Number	Gene	Primer Sequence 5'-3'
-	pSD5hsp60.MT3692 (F)	GGGCATCATATGCACGCTGTGACTCGTC
	pSD5hsp60.MT3692 (R)	GGGACGCGTTATTGATCGCTGATGGTCGATT
-	Kanamycin cassette (F)	GAGAAAACTCACCGAGGCAG
	Kanamycin cassette (R)	GTATTTCGTCTCGCTCAGGC
32287254	sigH (F)	GCGATGGTGGCTTCTCCCTCG
	sigH (R)	CCATCTTGCACAGCTCGCGTAG
11461	Mouse.β actin (F)	TAAGGCCAACCGTGAAAAGATG
	Mouse.β actin (R)	CTGGATGGCTACGTACATGGCT
21926	Mouse.TNF-α (F)	GACCCTCACACTCAGATCATC
	Mouse.TNF-α (R)	GCTGCTCCTCCACTTGGT
15977	Mouse.IFNβ (F)	CCACAGCCCTCTCCATCAAC
	Mouse.IFNβ (R)	CTCCGTCATCTCCATAGGGA
16193	Mouse.IL6 (F)	CTGCAAGAGACTTCCATCCAG
	Mouse.IL6 (R)	CAGGTCTGTTGGGAGTGG
16193	Mouse.IL-2 (F)	CCTGAGCAGGATGGAGAATTACAG
	Mouse.IL-2 (R)	CAATTCTGTGGCCTGCTTGGG
26827	Human. RNU6A (F)	CTCGCTTCGGCAGCACATATAC
	Human. RNU6A (R)	AATATGGAACGCTTCACGAATTTG
3456	Human.IFNβ (F)	CAACTTGCTTGGATTCCTACAAAG
	Human.IFNβ (R)	TATTCAAGCCTCCCATTCAATTG
3569	Human.IL6 (F)	GGTACATCCTCGACGGCATCT
	Human.IL6 (R)	GT GCCTCTTTGCTGCTTTCAC

6347	Human.MCP-1 (F)	GTGCTGACCC-CAATAAGGAA
	Human.MCP-1 (R)	TGAGGTGGTTGTGGAAAAGA
4843	Human.iNOS (F)	TGCAGACACGTGCGTTACTCC
	Human. iNOS (R)	GGTAGCCAGCATAGCGGATG
3586	Human.IL-10 (F)	GCTCCTGAGGTATGGAATAGAGTCC
	Human.IL-10 (R)	TATGTGTCATTTGCGGGGGC
64367	Rat.PPIB (F)	CAGGATTCATGTGCCAGGGT
	Rat.PPIB (R)	CCAAAGACCACATGCTTGCC
24481	Rat.IFN-β (F)	GAGTCTTCACACTCCTGGC
	Rat.IFN-β (R)	GTCCTTCAGGCATGAGACAG
298210	Rat.IFN-α (F)	GCGTTCCTGCTGTGCTTCTC
	Rat.IFN-α (R)	CCATTCAGCTGCCTCAGGAGC
25712	Rat.IFN-γ (F)	CGTCTTGGTTTTGCAGCTCT
	Rat.IFN-γ (R)	CGTCCTTTTGCCAGTTCCTC
24599	Rat. iNOS (F)	GGTGAGGGGACTGGACTTTTAG
	Rat. iNOS (R)	TTGTTGGGCTGGGAATAGCA
245920	Rat.IP10 (F)	TCCACCTCCCTTTACCCAGT
	Rat.IP10 (R)	AGAGCTAGGAGAGCCGTCAT
24770	Rat.MCP-1 (F)	CAGGTCTCTGTCACGCTTCTG
	Rat.MCP-1 (R)	GCCAGTGAATGAGTAGCAGCAG
25542	Rat.MIP-1α (F)	ACAAGCGCACCCTCTGTTAC
	Rat.MIP-1α (R)	GGTCAGGAAAATGACACCCG
116562	Rat.IL-2 (F)	CTGCAGCGTGTGTTGGATTT
	Rat.IL-2 (R)	GGCTCATCATCGAATTGGCAC
24494	Rat.IL-1β (F)	GACTTCACCATGGAACCCGT
	Rat.IL-1β (R)	GGAGACTGCCCATTCTCGAC
24835	Rat.TNF-α (F)	CGTCCCTCTCATACACTGG
	Rat.TNF-α(R)	CATGCTTTCCGTGCTCATG
59086	Rat.TGF-β (F)	TGACGTCACTGGAGTTGTCC
	Rat.TGF-β (R)	CCTCGACGTTTGGGACTGAT

25325	Rat.IL-10 (F)	CCTCTGGATACAGCTGCGAC
	Rat.IL-10 (R)	TGCCGGGTGGTTCAATTTTTC
24498	Rat.IL-6 (F)	GCGATGATGCACTGTCAG
	Rat.IL-6 (R)	GAACTCCAGAAGACCAGAGC
29221	Rat.Arg-1(F)	GGACATCGTGTACATCGGCT
	Rat.Arg-1(R)	GTAGCCGGGGTGAATACTGG

Table 4. Plasmids and Bacterial strains used in the study	
Name	**Description**
Plasmids	
pSD5.hsp60	Mycobacterial expression plasmid with hsp60 promoter
pSD5hsp60.MT3692	*disA* over-expression plasmid
***M. bovis* BCG strains**	
BCG	*M. bovis* BCG Pasteur
BCG-*disA* OE	BCG strain over expressing *disA* (MT3692)

Chapter 3

Generation of recombinant BCG overexpressing c-di-AMP

3.1 Introduction

Detection of invading pathogens by specific pathogen associated molecular patterns (PAMPs) by pattern recognition receptor (PRRs) is one of the earliest firewalls of the innate immune system. Initial encounter of *M.tb* by alveolar macrophages or dendritic cells initiates a series of innate immune responses. Diverse classes of germline-encoded PRRs associated with myeloid cells include toll like receptors such as TLR2 and TLR9, C-type lectin receptors, mannose receptors, scavenger receptors, NOD2 and NLRP3. NOD-like receptor family, AIM-2 like receptors (absence in myeloma, ALRs), cyclic GMP-AMP synthase (cGAS) and stimulator of IFN genes (STING), recognize specific PAMPs and thus promote activation of NF-κB, and type I interferon (IFN) responses and several downstream signaling networks. Downstream effects of activation of IRF-3 and NF-κB, lead to the transcription of innate effector molecules, including IFNβ, TNF, IFNγ, and IL1β [32,80]. These innate effector molecules are responsible for execution of a variety of antimicrobial effects, ranging from activation of cell death, such as apoptosis and autophagy in immune and stromal cells [81-83].

Previous studies from Bishai Laboratory showed that *M.tb* encodes di-adenylate cyclase enzyme, *disA* (MT3692) and can synthesize and secrete c-di-AMP. It was also demonstrated that c-di-AMP production and secretion was increased by over-expressing *disA* under a strong mycobacterial promoter, hsp60 [65,84]. Over-production of c-di-AMP allowed activation of the interferon regulatory factor (IRF) pathway and Type 1 IFN

responses through the (STING)-signaling pathway [85]. We wanted to test the therapeutic potential of wild-type vs recombinant BCG in a rat model of bladder cancer [79]. We hypothesize, that the BCG-*disA* OE strain due to its ability to activate STING-mediated Type I IFN responses will be more efficacious to conventional wild type BCG strain.

We decided to make changes to the genome of BCG Pasteur strain to improve its ability to overexpress c-di-AMP. We used similar strategy of overexpressing c-di-AMP that was employed earlier using *M.tb* strain as reported earlier [61]. Recombinant strains encoding and overexpressing *M.tb-disA* (MT3692) (BCG-*disA* OE) under strong bacterial promoter showed a robust increase in *disA* levels as compared to their wild-type parental control. Using *In vitro* infection experiments with murine reporter cell lines and primary bone marrow-derived macrophages (mBMDM) we found BCG-*disA* OE induced IRF activation coupled with augmented levels of IFNβ. We believe these strains could act like potential STING agonists and anti-tumor immunotherapeutic agents.

3.2 Results

3.2.1 Generation of recombinant BCG strain overexpressing *disA* (BCG-*disA* OE):

We generated a recombinant BCG strains (BCG-*disA* OE) overexpressing the endogenous di-adenylate cyclase (*disA*) encoded by gene MT3692 in the CDC1551 strain that facilitates c-di-AMP generation from ATP or ADP. MT3692 was cloned under strong promoter hsp60 and clones were confirmed using insert release followed by sequence analyses (Figure 1a and 1b). Recombinant clones were selected against kanamycin (25 µg/mL) using 7H11 plates. Single isolated colonies were picked and inoculated in the 7H9 media containing kanamycin (25 µg/mL). Recombinant strains were confirmed for the presence of kanamycin cassette (antibiotic selection marker) using colony PCR (Figure 1c). All clones,

except wild type BCG were positive for Kanamycin resistance (Figure 1c). Clones positive for the antibiotic selection marker were screened for *disA* overexpression phenotype using quantitative real-time PCR. Gene expression profiling showed ~50-fold upregulation of *disA* expression in BCG-*disA* OE as compared to their wild-type parental control (**Figure 2**).

3.2.2 Recombinant BCG overexpressing c-di-AMP results in IRF induction and IFN-β gene expression:

We next wanted to determine if the robust induction of *disA* expression in BCG-*disA* OE would result in c-di-AMP mediated induction of IRF3 and IFNβ as demonstrated previously [61]. Raw Blue™ ISG reporter macrophage cells were infected with wild-type and BCG-*disA* OE strains and the levels of secreted alkaline phosphatase (SEAP) was measured in the supernatant using QUANTI-Blue™. A 2-fold induction of IRF was observed in cells infected BCG-*disA* OE as compared to the wild-type control (**Figure 3**). Polyinosinic-polycytidylic acid or Poly (I:C) a synthetic analogue of double stranded RNA (dsRNA) and a molecular motif known to activate IRF3, was used a positive inducer of IRF in this assay [86]. Poly (I:C) mediated a strong induction of IRF in the assay **(Figure 3**). These results provide strong evidence that BCG-*disA* OE was producing heightened levels of c-di-AMP and activating of IRF in this reporter cell line. Next, we wanted to test the if BCG-*disA* OE could activate primary murine macrophages. Primary murine BMDMs were challenged with to wild-type BCG and BCG-*disA* OE strains for different time points and *Ifnb* induction was quantified using qPCR. A modest but significant induction of *Ifnb* was observed as early as 6 hours in BMDMs infected with BCG-*disA* OE as compared to wild-type BCG (**Figure 4**).

3.3 Discussion

Gene regulation in response to external stimuli and modification of cellular processes is enabled in organisms by signal transduction pathways, including CSPs. Second messenger moieties, such as cyclic di-nucleotides (CDNs) play prominent roles in growth, differentiation, and cellular stress responses in prokaryotes, including pathogenic insults and inflammation in metazoans [87].

Some of the key cyclic nucleotides such as, cyclic adenosine monophosphate (c-AMP), cyclic guanosine monophosphate (c-GMP), cyclic di-guanosine monophosphate (c-di-GMP), and cyclic di-adenosine monophosphate (c-di-AMP) can be classified as canonical as well as non-canonical CDNs. 2'-5' and 3'-5' cGMP-AMP are the natural products of cGAS (2'3' cGAMP) that have STING binding affinity owing to their mixed linkages and are referred to as non-canonical CDNs, while the CDNs associated with bacteria usually have canonical 3'-5' linkages [88].

Previously, our laboratory has shown that *M.tb* genome encodes a di-adenylate cyclase enzyme (*disA* or *dacA*, MT3692 in CDC 1551) that is involved in c-di-AMP synthesis. It was also shown that macrophages infected with *M.tb* strains overexpressing *disA* were activated by the bacterial-derived c-di-AMP resulting in the strong induction of IFNβ levels [61]. Since, c-di-AMP acted as a PAMP [80], this study was critical for understanding STING-mediated IRF and IFNβ induction. It was also found that increased levels of IFNβ was independent of interaction of c-di-AMP with cytosolic nucleic acid receptor cyclic-GMP-AMP (cGAMP) synthase (cGAS) [61].

Woodward et al. showed that bacterial infection of nonphagocytic cells leads to cytosolic penetration by leaky CDNs and that results in IFN induction [60]. Similarly, several studies

using intracellular pathogens such as *Chlamydia* and *Listeria* suggested STING-dependent induction of type I IFNs, that were dependent of CDNs greatly influenced virulence and intracellular survival in host cell niches [35, 60]. Orthologues of *disA* exist in all mycobacterial genomes except for *M. leprae*. The notion that CDNs can induce STING-dependent, but cGAS independent induction of type I IFNs (even in absence of extracellular pathogenic DNA) was validated by Dey et al., when they demonstrated that *disA* overexpressing strains of BCG were shown to induce a stronger Type I IFNs [61]. Since BCG strains lack the RD-1 locus or the Esx-1 secretion system required to release bacterial DNA, among other virulence factors, it was hypothesized that bacterial c-di-AMP was sensed by the macrophages leading to STING-dependent IFNβ synthesis. Additional experiments will be required to prove if BCG secretes c-di-AMP inside macrophages and exclude the possibility that cells are being activated because of dying bacilli and/or the leak of bacterial DNA from phagocytic vesicles.

Extracellular DNA of pathogenic bacteria and viruses (dsDNA) act as potent inducers of IFN α/β in infected macrophages [89]. The bacterial and viral nucleic acids are recognized by cytosolic cGAS for generation of cGAMP that in turn, bind and activate STING that subsequently phosphorylates adaptor protein TBK1. TBK1 phosphorylates IRF3 and initiates the transcription of several interferon stimulated genes (ISGs), including *Ifna, Ifnb* and other co-activated transcription factors, including the NF-kB and STAT6 [49, 90]. Previous studies with *disA* overexpressor strains of *M.tb* reported a strong co-activation of TNF, IL-6 and IL-1β in infected macrophages suggesting a possible cross-talk among different immune signaling pathways [65].

Our preliminary data with *disA* overexpressing strains suggested increased Type I IFN responses, a phenotype that was earlier reported in *M.tb*. This finding was first step towards generation of tools to be used as immunotherapeutic tool to treat NMIBC.

3.4 Figures

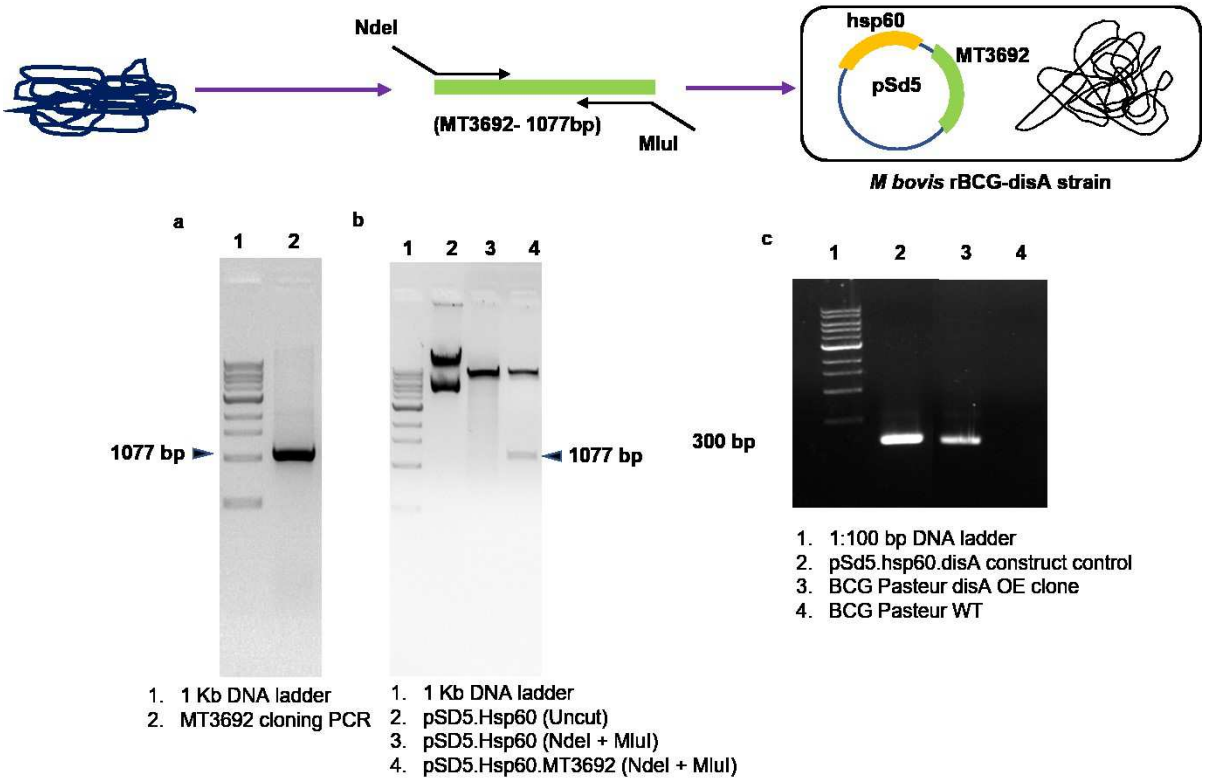

Figure 1. **Generation and confirmation of BCG Pasteur strain overexpressing *disA*.**

(A) The *disA* gene of M. tuberculosis, MT3692, which shows 100% homology with BCG *disA* gene was PCR purified from *M.tb* CDC 1551 genomic DNA using gene specific primers. **(B)** Gene amplicons and pSD5 vectors were separately digested using same set of restriction enzymes and were ligated at the NdeI and MluI restriction sites. This resulting ligated construct of pSd5.hsp60.MT3692 plasmid was used to transform BCG Pasteur strain and the recombinant clones were selected against Kanamycin (25 µg/mL). **(C)** Colony PCR using Kanamycin gene specific primers confirmed the presence of the recombinant plasmid in the BCG-*disA* OE strain.

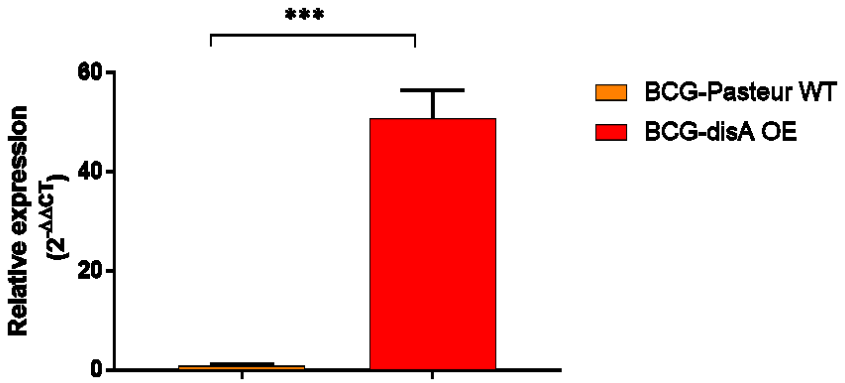

Figure 2. Overexpression of *disA* in BCG-*disA* OE strain:Quantitative real time PCR of BCG-Pasteur and BCG-*disA* OE strains for the *disA* gene (MT3692). For qPCR, total RNA was extracted from log phase BCG cultures, cDNA was prepared using SuperScript™ III First-Strand Synthesis. qPCR was performed using Applied Biosystems ABI StepOnePlus system. The expression was normalized first to the internal control (*sigH*) and then to wild-type BCG Pasteur. The graphical points represent mean of three independent experiments ± standard error mean (SEM). Student's t test (***P < 0.0005, **P<0.001).

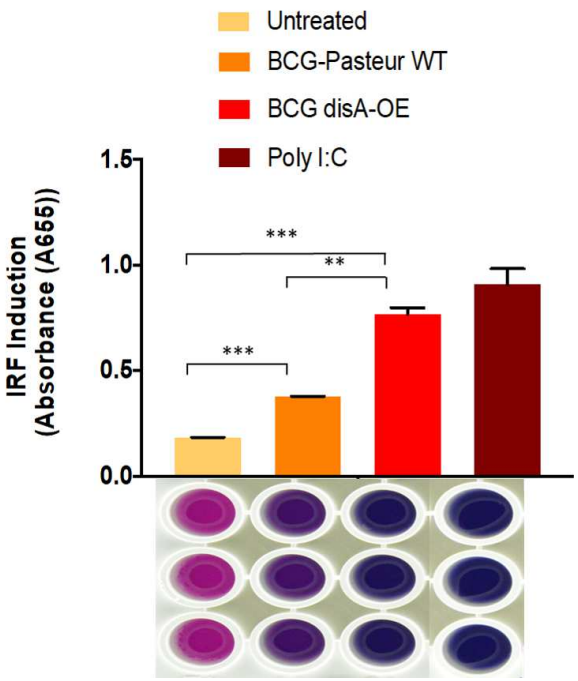

Figure 3. BCG overexpressing *disA* augments IRF3: Effect of *disA* overexpression on activation of IRF pathway measured by IRF-SEAP QUANTI Blue reporter assay. Mouse BMDMs were challenged with wild-type and BCG-*disA* OE strains at an MOI of 1:20 for 5 hours to establish the infection. Uninfected bacteria were washed out using ice-cold PBS and subsequently incubated for another 18 hours. The culture supernatants of infected RAW-Blue ISG cells were assayed for IRF activation. The image below the IRF-activation graph represents QUANTI Blue assay plate and sample wells; treatment parameters for column of wells correspond to those defined for the bars above aligned with the wells. The graphical points represent mean of three independent experiments ± standard error mean (SEM). Student's t test (***P < 0.0005, **P<0.001).

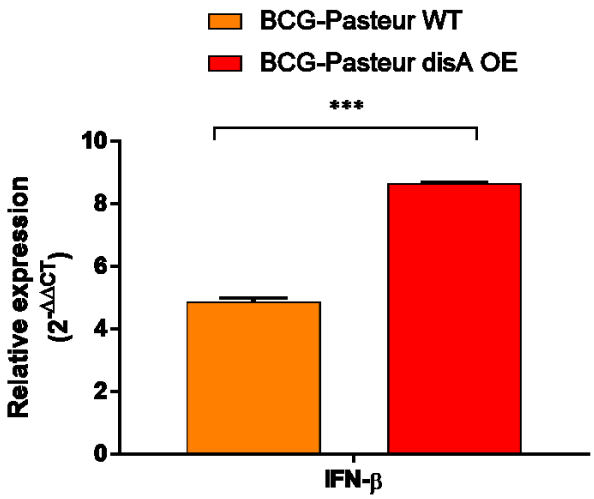

Figure 4. Differential expression of *Ifnb*: Mouse BMDMs were challenged with wild-type and BCG-*disA* OE strains at an MOI of 1:20 for 5 hours to establish the infection. Uninfected bacteria were washed using ice-cold PBS and cells were subsequently incubated for another 6 hours. Expression levels of mRNA was measured using a SYBR green-based quantitative real-time PCR. Basal level of transcript (mRNA) in untreated macrophages was used for data normalization and hence to access relative expression. β-actin was used as an internal control. Data analysis was performed using $2^{-\Delta\Delta CT}$ method. The graphical points represent mean of 3 independent experiments ± standard error mean (SEM). Student's t test (***P < 0.0005, **P<0.001). MOI (multiplicity of infection).

Chapter 4

In Vitro Immune Profiling in Macrophages against BCG-*disA* OE

4.1 Introduction:

STING was discovered by Glen Barber and colleagues as a MyD88-independent host cell defense factor expressed in macrophages, dendritic cells (DCs) and fibroblasts that induced expression of type I interferon-dependent and NF-κB dependent pro-inflammatory cytokines in response to cytoplasmic DNA [70,91]. Subsequently several additional DNA sensors were identified, such as Sox2, AIM2, DAI, IFI16 and DDX41, that can detect microbial DNA and induce innate immune responses, however, the cyclic GMP-AMP (cGAMP) synthase cGAS is considered the main sensor of cytosolic DNA [70,72,92-97]. Activation of cGAS/STING axis after recognition for foreign DNA leads to recruitment and activation of the kinase TBK1, which phosphorylates IRF3 and increases Type I IFN production. STING can also cross-talk with other transcription factors that are critical for synthesis of several cytokines and chemokines [49,90,97]. NF-kB, a heterodimeric transcription factor, and a key player of inflammatory and immune responses, is maintained inside the cytoplasm through its interaction with the inhibitory protein I kappa-B alpha (IκBα). Upon cytokine stimulation, a multi-subunit protein kinase, the I kappa B kinase (IKK), is rapidly activated and phosphorylates two key serine residues in the N-terminal regulatory domain of the IκBα, that results in the separation of the NF-kB dimer and degradation of IκBα by the proteasome. The Rel A/p50 NF-kB dimer then translocates to the cell nucleus and mediates inflammatory responses [99]. STING can also directly

activate Iκκ, which in in turn phosphorylates the inhibitor IκBα to release NF-κB and influence gene transcription of pro-inflammatory cytokines [100]. Although, the binding affinity of c-di-AMP for STING is far weaker than cGAMP, ligation of c-di-AMP to STING appears sufficient to induce co-activation of transcription factors other than IRF3 and hence induce pro-inflammatory signatures [58,101]. Identification of another bona-fide physiological sensors for c-di-AMP, an endoplasmic adaptor, ERAdP, that binds to c-di-AMP with high affinity, results in initiation of activation of NF-κB signaling in innate immune cells to induce the production of pro-inflammatory cytokines during bacterial infection indicating a rather complex link between c-di-AMP-based STING activation and induction of Type I IFN and pro-inflammatory cytokines [102]. Nevertheless, direct activation of NF-kB by cGAS/STING/TBK1 axis is sufficient to explain induced levels of proinflammatory cytokines along with interferon α/β in immune cells due to increased levels of canonical or non-canonical CDNs [101,103-105].

M.tb strains engineered to overexpress *disA* induce several key pro-inflammatory cytokines, including IL-6, TNF and IL-1β. We used gene expression and cytokine secretion to determine the immune profiles, characteristic macrophages infected with wild-type or BCG-*disA* OE. Previously, we demonstrated that BCG-*disA* OE strain increased IRF3 induction of and *Ifnb* expression in macrophages when compared to the wild type control BCG (**Chapter 3, Figure 4**) In addition, we profiled immune responses in macrophage cell lines and primary macrophages at the transcript as well as the protein level using qPCR and ELISA, respectively. In the work presented in this chapter, immune profiling using qPCR assay showed a distinct initial wave of STING-induced effector molecules (cytokine and chemokine) in macrophages infected with BCG-*disA* OE. ELISA results corroborated

gene expression profile suggesting true biological modulation of immune effectors. We found increased expression of key Th1 (IFNy and IL-2) and M1 cytokine (TNF and IL-6) in macrophages infected with BCG-*disA* OE strain, as early as 3 hours after infection. A potent increased in cytokines (TNF, IL-6 and IL-1β) and chemokines (MCP-1) were also observed in macrophages infected with BCG-*disA* OE of BCG. This *in vitro* immune profiling strategy to identify the characteristic immune responses in macrophages in response to increased level of intracellular c-di-AMP suggested that the CDN c-di-AMP potently activates the CSP of macrophages. Also, these results suggest that BCG-*disA* OE strain functions as a potent STING agonist and has potential as a potential immunotherapeutic tool against certain forms of cancer.

4.2 Results:

4.2.1 Immune profiling of macrophages infected with wild-type or BCG-*disA* OE: We challenged C57BL/6J BMDMs with wild-type BCG or BCG-*disA* OE strain to identify differential gene expression profile of key cytokines at an early temporal window. Since we expected a rapid and change in macrophage environment in response to enhanced *disA* transcription and c-di-AMP production, an early time point was selected for gene expression profiling. A rapid nuclear translocation of RelA/p50 subunits and hence induction of gene expression is a hallmark of NF-κB signaling during pathogenic insult. Uninfected macrophages were considered controls for basal level gene expression, while macrophages infected with wild type BCG were considered the infection control to determine because the impact of *disA* overexpression. We found increased abundance in the number of transcripts for the genes encoding TNF, IL-6 and IL-2 as early as 5 hours after the macrophages were infected with BCG-*disA* OE compared to the wild-type BCG

(Figure 5). Increases in TNF-α and IL-6 have been previously reported in macrophage infected with *M.tb disA* OE infected macrophages as compared to those infected with wild-type parental controls [77]. The differential induction of Th1 (IL-2) and M1 (TNF and IL-6) cytokines have been hypothesized as direct consequence of coactivation of NF-κB, and STAT6 transcription that follows or parallels IRF3 activation in response to CDNs [49].

We next ascertained the translatability of gene expression profile by performing ELISA using the culture supernatants from macrophages infected with wild-type or BCG-*disA* OE strains of BCG. Two different cell types namely, mBMDM and human THP-1 cells were used perform *in vitro* cytokine ELISA. Resting mBMDMs were challenged with wild type and BCG-*disA* OE strains, culture supernatants were harvested at 24 hours and were quantified using ELISA based assessment of cytokine production. We found significantly increased levels of TNF, IL-6 and IL-1β in murine BMDMs infected with BCG-*disA* OE strains as compared to those infected with wild-type parental BCG (Figure 6) suggesting macrophage polarization towards M1 phenotype. This immune activation phenotypes observed in mouse-derived cells was also seen in human derived, PMA differentiated, IFN-γ activated THP-1 cells. Increased levels of IL-6, MCP-1 and IL-1β in response of *disA* overexpression (Figure 7) clearly suggested a coactivation NF-κB and STAT6 that has been proposed once STING is activated by CDNs [90,106]. STAT6 plays a prominent role in adaptive immunity by transducing signals from extracellular cytokines. DNA viruses are known to induce STING and recruit STAT6 at the endoplasmic reticulum (ER) site leading to phosphorylation of specific Serine residues at STAT6 by TBK1, that is independent of JAKs [90,107]. This mechanism is crucial for nuclear translocation of STAT6 and transcription of specific target genes responsible for immune cell (monocytes,

macrophages and T cells) homing, most importantly MCP-1 [90,108-109]. Recent studies by Lalita Ramakrishnan's group showed PGL-1 dependent STING activation is responsible for MCP-1 or CCL2 secretion in human alveolar macrophages [110]. CCL2 is important for recruitment of inflammatory monocytes to areas of inflammation and acts like a bridge between innate and adaptive immunity. We tested the differential modulation pattern of MCP-1/CCL2 in human macrophages and found a strong induction of CCL2 in culture supernatants of macrophages infected with *disA* OE infected macrophages (Figure 7)

4.3 Discussion:

Canonical and non-canonical cyclic di-nucleotides (CDNs) are undergoing clinical testing to determine their ability to activate STING/TBK1/IRF3 axis and generate a strong anti-tumor response (**Table 1**). This new class of STING agonists have proven efficacious in *in vivo* models of non-immunogenic and immunogenic tumors [35-36]. Treatment of NMIBC bladder cancer is based on BCG-mediated cancer immunotherapy and STING agonists have also been tried using different models to prove their effectiveness [111, 112]. Our aim was to generate a BCG strain that not only carries the immune-dominant epitopes of mycobacteria but also selectively activates STING-dependent anticancer Type I interferons. Although we found increased IFNβ, we also found increased pro-inflammatory cytokine such as TNF, IL-6 and IL-1β, suggesting a possible augmentation of NF-κB and STAT signaling that is a result of co-activation either through autocrine or paracrine mechanisms.

BCG strains lack the Esx-1 secretion system and hence BCG is thought to be unable to release bacterial DNA inside macrophages [89]. Recently these models of ESAT-6-dependent changes in vacuolar phagosome has been challenged [65]. *M.tb* containing phagosomes are considered a dynamic and relatively leaky structure that can facilitate communication between macrophage cytosol and phagosome harboring bugs. Other studies suggest leakage of c-di-nucleotides (CDNs) in adjacent cells through gap junction, a mechanism responsible for induction of pathogen-specific IFNα/β secretion that provides immunity to the invading pathogens [60]. We generated a recombinant BCG strain that caused strong induction of *M.tb disA* (**Figure 2, Chapter3**), activated IRF induction and IFNβ in murine macrophages. Whether induction of IFNβ was dependent on STING/TBK-1 axis and due to increased influx of c-di-AMP inside macrophages remains to be validated by direct measurement of c-di-AMP levels inside macrophages. However, we observed stronger proinflammatory cytokine signatures that were very much like studies that have been performed using STING agonist or by directly inducing increased c-di-AMP levels inside macrophages [49]. Our results suggest a secondary effect of STING activation that can canonically or non-canonically activate NF-κB and STAT6. These pro-inflammatory cytokines can further activate macrophages in an autocrine or paracrine manner [113]. Increased levels of CCL2 in culture supernatants derived from BCG-*disA* OE infected macrophages could possibly be due to STAT6 activation. We yet do not know the exact mechanism of induction of CCL2 and immune activated phenotype, but, it will be interesting to test these phenotypes using cancer models at the site of tumor microenvironment (TME). Interestingly, a recent study clearly showed that STING agonist (ADU S100) showed antitumor function through activation of key pro-inflammatory

cytokines, including TNF, IL-6, IL-1β and chemokine such as CCL2/MCP-1 and IP10 at the site of drug administration [49]. It is also worth mentioning that these cytokines contributed not just by recruiting monocytes and macrophages but also by activating stromal cells [49]. Overall, we generated a BCG strain that was a not just a potent inducer of Type I IFN but also caused a bias towards pro-inflammatory cytokine production. These findings provided us with a clear clue of immunotherapeutic potential of these strains.

4.4 Figures

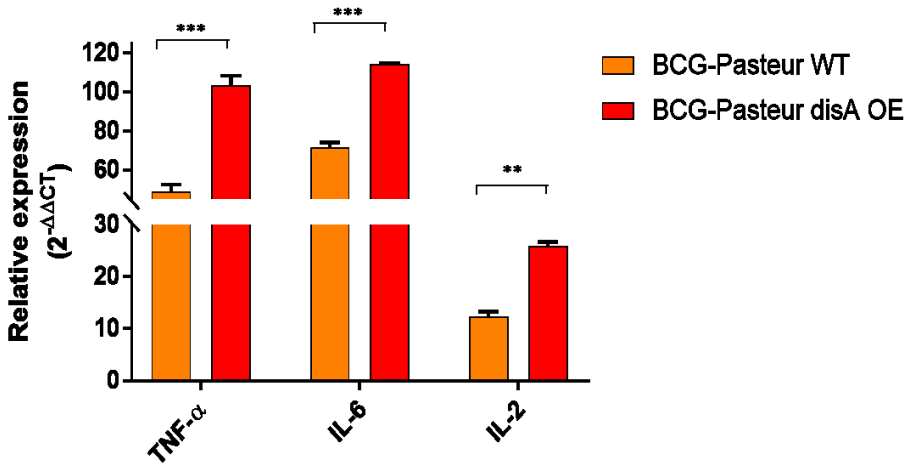

Figure 5. BCG overexpressing *disA* expresses pro-inflammatory cytokines in BMDMs. Differential expression of genes encoding TNF, IL-6 and IL-2 in mouse BMDMs challenged with wild-type and *disA* overexpression strains of Mycobacterium bovis BCG-Pasteur. BMDMs were challenged with wild-type and *disA* OE strains at an MOI of 1:20 for 5 hours to establish the infection. Uninfected bacteria were washed using ice-cold PBS and cells were subsequently incubated for another 6 hours. Expression levels of mRNA was measured using a SYBR green-based quantitative real-time PCR. Basal level of transcript (mRNA) in untreated macrophages was used for data normalization and hence to access relative expression. β-actin was used as an internal control. Data analysis was performed using $2^{-\Delta\Delta CT}$ method. The graphical points represent mean of three independent experiments ± standard error mean (SEM). Student's t test (*P < 0.05 **P < 0.01, ***P < 0.001). MOI (multiplicity of infection).

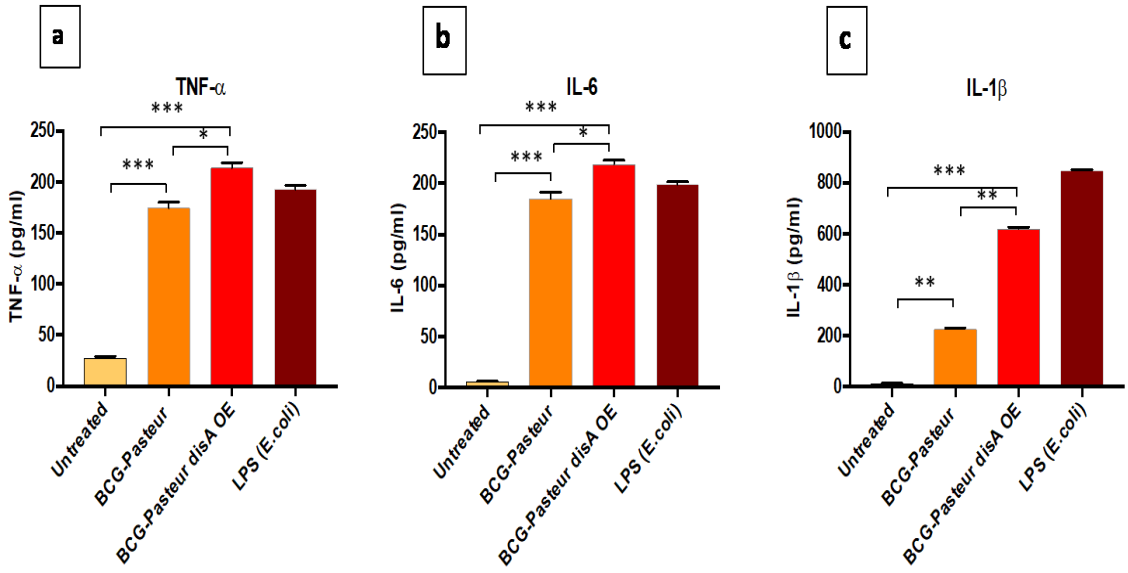

Figure 6. Increased pro-inflammatory cytokines in response to *disA* overexpression in BMDMs. Differential expression of TNF **(A)**, IL-6 **(B)** and IL-1*β* **(C)** in mouse BMDMs challenged with wild-type and *disA* overexpression strains of *Mycobacterium bovis* BCG-Pasteur. BMDMs were challenged with wild-type and *disA* OE strains at an MOI of 1:20 for 3 hours to establish the infection. Uninfected bacteria were washed using ice-cold PBS and cells were subsequently incubated for another 18 hours. Culture supernatants were assayed by ELISA for different cytokines. The graphical points represent mean of three independent experiments ± standard error mean (SEM). Student's t test (*P < 0.05 **P < 0.01, ***P < 0.001). MOI (multiplicity of infection).

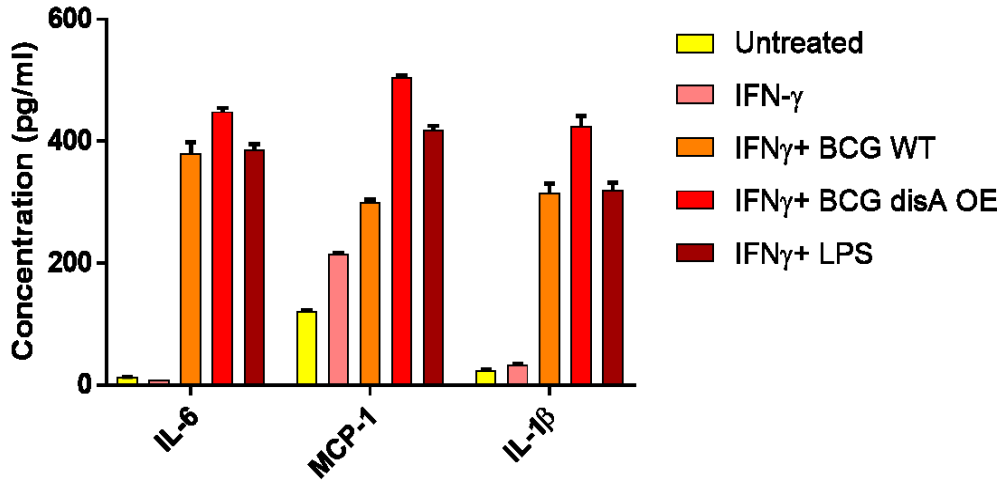

Figure 7. Increased pro-inflammatory cytokines in response to *disA* overexpression in human THP-1 cells. Differential expression of IL-6, MCP-1 and IL-1β in PMA-differentiated THP-1 cells stimulated with IFN-γ for 6 h followed by challenge with wild-type and *disA* overexpression strains of *Mycobacterium bovis* BCG-Pasteur. THP-1 cells were challenged with wild-type and *disA* OE strains at an MOI of 1:20 for 5 hours to establish the infection. Uninfected bacteria were washed using ice-cold PBS and cells were subsequently incubated for another 18 hours. Culture supernatants were assayed by ELISA for different cytokines. The graphical points represent mean of three independent experiments ± standard error mean (SEM). Student's t test (*P < 0.05 **P < 0.01, ***P < 0.001). MOI (multiplicity of infection).

Chapter 5

Therapeutic efficacy of BCG-*disA* OE c-di-AMP overexpressing strains and anti-tumor responses in a rat model of urothelial carcinoma

5.1 Introduction:

BCG has remained mainstay of bladder cancer immunotherapy over the last 40 years [114]. There is no consensus on a universal mechanism of BCG action, but a complex immunomodulation ensues after BCG instillation. BCG attaching to urothelial cells, including urothelial carcinoma cells that results in the secretion of an array of pro-inflammatory cytokines (TNF, IL-6, IL-1β and IL-8) and chemokines (GM-CSF/ CFS2), that causes urothelial cell activation [115]. This activation is followed by an influx of neutrophils, monocytes, macrophages, lymphocytes, NK cells, and DCs. The infiltrated cells become activated and contribute to a variety of additional pro-inflammatory cytokines and chemokines [116]. This results in transmigration of large number of neutrophils, T cells and macrophages into the bladder lumen where they can be readily measured in the patient's urine. Patients receiving BCG instillation have a transient and massive cytokine and chemokine flux that appears in urine that includes elevated IL-1β, IL-2, IL-6, IL-10, IL-12, IL-18, IFNγ, TNF, GM-CSF/CSF2, macrophage colony-stimulating factor (M-CSF/CSF1), macrophage-derived chemokine (MDC/CCL22), monocyte chemoattractant protein 1 (MCP-1/CCL2), macrophage inflammatory-protein-1α (MIP-1α/CCL3), interferon-inducible protein (IP-10/CXCR3/MIG) and eosinophil chemoattractant activity (Eotaxin/CCL11) [117-119]. *In vitro* assays suggest both human and murine macrophages

play tumoricidal roles against bladder cancer cells upon BCG stimulation [120-121]. Macrophage mediated killing of bladder cells depends on direct cell to cell contact and release of various cytotoxic cytokines such as TNF and IFNγ and apoptotic mediators such as nitric oxide (NO) as well as direct cytotoxicity mediated by infiltrating lymphocytes [55,56,122-124]. Clinical studies reveal that a predominant Th1 cytokine profile (e.g. IFN-γ, IL-2, and IL-12) is linked to therapeutic effects of BCG, whereas a high level of Th2 cytokine such as IL-10 and arginase-1 is linked to BCG failure, suggesting a shift from Th2 to Th1 environment for a successful therapy [125-127]. There is a gradual loss of BCG induced immune response after the initial treatment, which explain the need for maintenance therapy to boost cytokine response and immune infiltration [128]. Also the beneficial effect of BCG priming on BCG instillation, is directly linked to improved inflammatory responses and recruitment of T cells to the bladder in healthy animals compared to a mere BCG single-dose instillation [129].

Type I IFN were characterized in late 1970s from the culture supernatants of human leucocytes exposed to viruses that was followed by synthesis of IFNα2a and IFNα2b in 1980s as unmodified recombinant proteins or as their PEGylated versions with improved self-life. Interferons were simultaneously approved by various regulatory agencies for the treatment of multiple neoplasms [130]. PEGylated IFNα2b has been used for the treatment of resected stage II and III melanomas [131]. Type I interferons favor the influx of dendritic cells (DCs) and T cells into neoplastic lesions in patient suffering from ulcerated primary tumors [132-133]. Despite the beneficial effects of Type I IFN, the treatment is accompanied by several undesirable systemic adverse outcomes, and hence, attempts began to specifically deliver Type I IFN to the tumor microenvironment [134]. Type I IFNs

conjugated to monoclonal antibodies (MAb) such as C22b2b (tetrameric IFNα2b coupled to hL243 (a humanized monoclonal antibody that is specific for HLADR), and IFNβ fused to cetuximab were some of the modifications that were tested against human myeloma and EGFR expressing tumors respectively [130,135]. Another approach was generation of Type I IFN encoding vectors of various types (e.g. adenoviruses) that were directly injected into tumors. An adenovirus encoding IFN-α reduced intratumoral abundance of Tregs and promoted accumulation of TH17 cells in CT26 colorectal carcinomas in a mouse model, most likely due to increased IL-6 produced by recruited CD11c+DCs [136].

Type I IFN in combination with BCG immunotherapy has shown significant anti-proliferative and immunomodulatory effects than BCG monotherapy itself. Using mouse model of bladder cancer, 14/15 mice receiving BCG/IFNα versus 8/15 mice receiving only BCG became tumor-free after 5 weekly intralesional treatments [137]. In an interesting study, peripheral blood mononuclear cell (PBMC) cultures showed ~66-fold increased IFNγ production when challenged with BCG plus IFNα compared to BCG alone [115]. IFNγ is a major Th1-restricted cytokine found in patients responding to BCG immunotherapy, it has been routinely used as a surrogate marker for Th1 immune response [51]. IFNα itself generates an almost negligible effect on IFNγ levels, but BCG-IFNα combinatorial treatment, even at lower dosage of BCG was enough to induce increased and persistent IFNγ levels, along with other pro-inflammatory cytokines (TNF and IL-12) [51]. This study supports the idea that adding Th1-stimulating cytokine might reduce dosage of BCG immunotherapy and hence associated side effects.

Type I IFN plus BCG immunotherapy has shown promises for BCG refractory patients. The combination therapy appeared safe with a similar side effect profile of BCG

alone [138]. In a remarkable study, O'Donnell and colleagues showed that a combination therapy administered to 40 patients who failed at least one course of BCG alone, was effective to cure 53% patients after 24 months of treatment [139]. There has been a concerted effort by the research community to make desirable modifications of BCG that can be used as a cocktail of immunotherapeutic cytokines (**Table 2, Chapter 1**). Recombinant BCG strain (rBCG-IFNα) expressing IFNα2b enhanced IFNγ expression in PBMCs after incubation with rBCG-IFNα as compared to standard BCG, additionally it showed increased cytotoxicity in PBMCs as compared to BCG alone most likely due to $CD56^{+}CD8^{-}$NK cells and $CD8^{+}$ T cells [140]. New developments include generation of BCG strains capable of secreting IFNα2b, that cause exuberant immune response and cytotoxicity against human bladder cancer cells. Therapeutic benefits of Type I IFN based anti-tumor immunotherapy relies on its direct cytotoxicity effects on tumors and its ability to promote maturation of dendritic cells (DCs), antigen presentation, and enhance NK or $CD8^{+}$ T-cell mediated antitumor immunity [116].

Recently, STING has been proposed to be the key player against immunogenic tumors [35,141]. Mice lacking STING experience uncontrolled growth of transplanted tumors as compared to their wild-type counterparts. Similarly, STING-deficient mice show spontaneous CD8+T cell priming against tumors but not in animals with missing TLRs, MyD88 or MAVS adaptor suggesting important role of cytosolic immune surveillance in tumor immunity [35]. Tumor-derived DNA is an important player for STING activation, Type I IFN production and DC-mediated cross-priming, the immunological events critical for generating anti-tumor adaptive immunity [142].

Since STING activation by tumor-derived DNA appears to play a role in tumor growth; targeting STING by using a STING agonist for augmented antitumor response appears to be a promising cancer therapy. Several STING agonists are under different stages of clinical development (**Table 1, Chapter 1**) for specific cancer sub-types. Direct delivery (intra-tumoral administration) of cyclic dinucleotides (e.g. cGAMP, c-di-GMP, and c-di-AMP) into tumor bearing mice has shown substantial growth inhibition of tumors due to activation of STING-mediated TBK1/IRF3/IFN axis [49]. Although STING agonists are promising antitumor agents, there are challenges, such as development of tolerogenic responses and hence increased tumor size due to STING [143]. The leaky nature of CDNs (cGAMP can cross cell to cell via gap junctions), is another concern related to in vivo application of STING agonists as it might increase pro-inflammatory cytokines and promote tumor growth. Still, optimal combination of STING agonists with other treatment regimens such as chemotherapy and radiation therapy, and blockade of immune checkpoint inhibitors should be developed and tested.

BCG treatment cures almost 70% of the bladder cancer patients and BCG is a promising and effective antigen delivery tool [127]. Several studies corroborate an underlying immune response skewed towards Type I IFN and Th1 is associated with a positive treatment outcome and hence there is an ongoing effort to develop BCG strains that express human Type I cytokines, but till date such efforts have not yielded demonstrable improvement over traditional BCG. Resistance to BCG treatment and disease recurrence in a significant proportion of patients with NMIBC prompts the need to improve the efficacy of BCG [53-56, 115]

Previous studies published from our laboratory showed *M.tb* strains overexpressing c-di-AMP can induce excessive IFNβ and pro-inflammatory cytokines such as TNF and IL-6. We have generated a BCG strain overexpressing bacterial PAMP, c-di-AMP (rBCG-*disA* OE) that generated Type I IFNs in mouse and human macrophages (**Chapter 2 and 3**). We next tested the immune responses elicited by both wild-type and *disA* OE of BCG in different human and rat bladder cancer cells before testing these strains using a relevant *in vivo* animal model of bladder cancer.

Animals used as a model of bladder cancer can vary from large mammalian animals including dogs or monkeys which recapitulate the human disease but have many financial and ethical issues associated, to rodents (rats and mice) [146]. Rodents breed well, reproduce, less expensive to be maintained and can allow a lot of variation to be studied. In reported literature both rats and mice have been equally employed as models of bladder cancer. For example, in carcinogen induced model rats are more frequently used due to the advantage of size and possibility of intravesical treatment (specially in MNU induced tumors) however in studies requiring the characterize the underlying genetics of the cancer mice have been preferred owing to the higher numbers required. Furthermore, in the rodents, there is an underlying difference in the pathological and structural characteristics of bladder tumor pathogenesis. In rat induced tumors, the tumors are often pedunculated and with an inverted papillary growth pattern and characterized as papillary neoplasms [147]. While mice usually form more nodular hyperplasia's which are less frequently seen in humans as compared to papillary neoplasms [148]. The rat has been used most frequently in these studies to take advantage of the larger body/organ size as in modelling intravesical treatment and catheterization [149-150]. Bladder cancers induced in rats also exhibit

histopathologic and immunophenotypic features similar to those observed in human urothelial cancer. Genetic homology comparisons show that human and rat genes are 90% homologous, compared to 80% homology between human and mice genes [149-150].

Considering the advantages of using a rat rodent model and orthoptic chemical carcinogenic MNU instillations, the Bivalacqua lab pioneered a Fischer rat model for NMIBC. In this model, urothelial dysplasia develops within 8 weeks of MNU instillation, and by the 16^{th} week after the first instillation, all rats display carcinoma in situ, papillary Ta, or high-grade T1 urothelial carcinoma with histopathologic and immunophenotypic features similar to those observed in human urothelial cancer [79]. Using this model, the Bivalacqua lab showed that intravesical delivery of BCG immunotherapy led to a large, transient rise in the $CD4^+$ T cell population in the urothelium [79].

We earlier found that the BCG strain overexpressing c-di-AMP, induced a potent Type 1 IFN response along with M1 type cytokine in murine and human macrophages. We first decided to test the immune responses against BCG-*disA* OE in different human and rat cancer cell lines to define the immune responses *in vitro*. BCG-*disA* OE when incubated with different stage-specific cell lines showed increased M1 cytokines, and increased IFN levels. We next wanted to test the therapeutic potential of BCG-*disA* OE in the MNU-induced rodent model of bladder cancer. This was achieved via direct instillation of wild-type or BCG-*disA* OE strains to determine the impact of treatment outcome on cancerous bladders. By performing an extensive panel of cytokine and chemokine profiling coupled with histopathological analyses we demonstrated the relative efficacy of c-di-AMP over expressing BCG strains in tumor bearing bladders. Although our results clearly show treatment benefits, key questions related to immunological memory responses,

contribution of T cell activation, cellular infiltration and expansion and contribution of myeloid cells remains to be understood in the *in vivo* model.

5.2 Results and Discussions

5.2.1 BCG-*disA* OE overexpressing c-di-AMP induces potent Type I IFN coupled with enhanced anti-tumor responses in different bladder cancer cells:

Human transitional cell papilloma RT4 bladder cancer cells, 5637, a human grade II carcinoma and NBT-II *Rattus norvegicus* cell line (derived from a tumor induced with N-butyl-N-(4-hydroxybutyl) nitrosamine) were used to test immune responses after challenge with BCG-*disA* OE strain and directly compare it with immune responses against wild-type BCG. Cell lines used here represent different stages of bladder cancer and yield information on any notable differences in the physiological response.

One of the most striking effect of c-di-AMP overexpression was strong induction of *Ifnb* transcription in 5637, RT4 and NBT-II cells as early as 5 hours after BCG exposure (Figure 8). As previously stated, STING-mediated IFNβ induction correlates tumor control and anti-tumor milieu in the tumor microenvironment (TME) and increased IFNβ levels promotes efficient cross-talk between innate and adaptive immunity [134]. These results hint that BCG-*disA* OE is a stronger inducer of anti-tumor type I IFNs.

We next looked into induction of Th1 cytokines. And found a strong induction of IFNγ in NBT-II cancer cells (~2-fold as compared to wild-type treated cells) (Figure 11). Earlier reports demonstrated that IFNγ, takes the lead in dictating the cellular anti-tumor response and that an increase in IFNγ is associated with effective anti-tumor responses [151]. Our

in vitro experiments suggested increased IFNγ, another key anti-tumor cytokine and activators of macrophages.

Macrophages and neutrophils are usually the first cells to accumulate in the bladder after BCG instillation in response to the type 1 cytokine environment. Major M1 immune effectors cytokines TNF and IL-6 are reported to be induced downstream of STING-dependent NF-κB activation in myeloid as wells as stromal cells [49]. TNF and IL-6 showed a modest increase in the carcinogen induced rat bladder cancer cells (NBT-II) (Figure 11), while a significant increase in IL-6 was observed in Human grade II carcinoma cells (5637) (Figure 10). The increased induction of IL-6 was further corroborated by ELISA-based quantification of the secreted cytokine in the culture supernatants 24 hours after treatment with BCG strains (Figure 12). There was insignificant change in amount of IL-6 in RT4 cell lines (Figure 12), most importantly, we found a very similar immunological response in RT4 cells in response to BCG infections as reported elsewhere [152-153].Cytotoxic T cells are important in tumor cell killing, an event attributed to T cell recruitment (infiltration) that depends on endothelial tumor cells secreting CCL2/MCP-1 [154]. Human PBMCs, bladder cancer cells and PBMCs from BCG vaccinated individuals when challenged with BCG triggered a significant increase in key chemokines and chemotactic factors for the recruitment of myeloid cells and lymphocytes to the site. These chemokines included MCP-1/CCL2, MIP-1α/CCL3 and IP-10/CXCL3. We observed a similar increase in these chemokines quantified at the site of the tumors treated with BCG [152]. We found a marked increase in the transcription (Figure 9 and 11) and protein production (Figure 12 of CCl2, MIP-1α and IP-10) in different bladder cancer cell types when exposed to BCG-*disA* OE strain compared to controls.

Juan Fu et al. measured the efficacy of synthetic CDNs as a STING agonist for their efficacy in reactivity against PD-1 resistant tumors in an *in vivo* and *ex vivo* mouse for colon, tongue, liver and pancreatic carcinomas [36]. They observed a characteristic NF-κB-dependent TNF, IL-6, and STAT6-dependent MCP-1/CCL2 cytokines in murine BMDMs. We found a significant induction of MCP-1/CCL2 in RT4 and NBT-II cells (Figures 9 and 11). Further, it was elucidated in cell culture supernatants treated with BCG strains, that STING activation in the bladder cancer cells induces MCP-1/CCL2 protein in 5637 cells (Figure 12). However, RT4 human papilloma cell lines failed to express any MCP-1/CCL2 protein in cell culture supernatants collected after 24 hours. Key chemokines which have been reportedly associated with increased accumulation of adaptive and effector cell types are IP-10/CXCL3 and MIP-1α/CCL3 [152] which was generally observed to have increased in the case of BCG treatment in Bladder cancer seemed to also be induced higher in the rat bladder carcinoma cell lines (Figure 11).

Early on in 1996, clinical studies on IP-10/CXCL3 in human non-small cell lung cancer had been indicated to be a angiostatic factor which inhibited tumorigenesis and spontaneous metastases [155]. Further IP-10/CXCL3 has been shown to be significantly increase in the urine of BCG immunotherapeutic received bladder cancer patients. BCG-*disA* OE infections caused differential increased in IP-10 in the NBT-II cell lines (Figure 11) [156]. MIP-1α/CCL3 which is also significantly increased in response to BCG-*disA* OE in the rat bladder carcinoma cell lines, is known to be a prognostic marker for BCG immunotherapy in NMIBC (Figure 11).

Another prominent M1 cytokine which has shown a strong anti-tumor activity in different cancers and *in vivo* and *in vitro* models is Nitric oxide (NO). In a murine model, it has been

shown in pancreatic carcinoma that stromal cells in tumor microenvironment release higher NO which is iNOS mediated that is associated with anti-tumor activity. [164] Increased relative levels of transcription of the gene encoding iNOS by BCG-*disA* OE was also seen in all the grades and origin bladder cancer cell lines in our study (Figure 9, 10 and 11).

Previous studies done in the Bivalalqua laboratory, have shown that wild type BCG in their MNU induced rat model increased infiltration and expansion of $CD4^+$ T cells [79]. Another axis which has shown to increase in bladder cancer cell lines stimulated with BCG-*disA* OE is the inflammasome/IL-1β pathway [157]. Downstream of IL-1β signaling several chemokines are induced including CCL2, CCL5 and CXCL12 [158]. Of these CCL2 has been extensively shown to be the reason for infiltration and recruitment of myeloid cells to the site of inflammation [159]. Further, in a study in an orthoptic model of murine breast carcinoma, it has also been shown that IL-1β-induces CCL2 expression in macrophages and tumor cells [160]. However, due to the conflicting literature on the role of increased IL-1β leading to a more tumorigenic microenvironment, more investigation is required in to this axis in the context of bladder cancer [161]. Also, a report by Gaidt et al. posited that STING activation can result in targeting a DNA inflammasome as one of the downstream targets which can also lead to induction of IL-1β as a co stimulatory effect [162]. We observed an increased expression of IL-1β in all the three cell lines stimulated by BCG-*disA* OE as compared to the WT strain (Figures 9, 10 and 11.), and there was a commensurate increase in IL-1β protein secretion into cell culture supernatants in 5637 cells (Figure 12). However, like IL-6 and MCP-1/CCL2 responses, differential IL-1β protein production was not seen in RT4 cells (Figure 12). The increased pro-inflammatory microenvironment in cancer cells could potentially contribute to massive recruitment and

activation of macrophages and monocytes that can contribute to anti-tumor responses. Our results suggest a very similar immune profile as suggested earlier when STING agonists are administered at the site of tumor.

IL-10 gene expression was quantified in the human 5637 (Figure 10) and rat cell line NBT-II (Figure 11). Notably, IL-10 gene expression was repressed in both cell lines with a lack of transcription in the group treated with BCG-*disA* OE (Figure 11). IL-10 plays an antagonistic role to the IFNβ in BCG induced macrophage cytotoxicity to Bladder cancer cell lines [163].

5.2.2 Therapeutic efficacy of the BCG-*disA* OE overexpressing c-di-AMP in a MNU induced Fischer Rat model of bladder cancer: In the Bivalacqua laboratory, the MNU-Fischer rat model of bladder cancer was first pioneered and characterized in 2017 [79]. They showed that MNU-Fischer rat model of bladder cancer to have a similar pathology and immunophenotype to that of human bladder cancer.

We used a similar intravesical instillation approach for BCG-*disA* OE strain vs BCG WT strain to test the therapeutic efficacy of the recombinant strain (Figure 13). The readout of this pilot study was histopathological analysis and scoring of tumor involvement index based on the tumor grade and spread in the urothelium. To monitor the effects of the *disA* overexpression immune markers were also measured at the site of tumor induction and BCG instillation. At the end of the 16th week after the first instillation, serial ultrasound examinations revealed the development of papillary tumors. All specimens (n = 5) at week 16 had evidence of CIS, papillary Ta, or high- grade T1 urothelial carcinomas (Figure 13c) consistent with the 2004 World Health Organization/International Society of Urological Pathology classification system. After intravesical instillation of BCG-*disA* OE, pathologic

analysis of the bladder showed a lowered tumor involvement index in the tumors that were generated in the BCG-*disA* OE strain treated animals as compared to the wild type BCG Pasteur strain. ($P < 0.05$) (Figure 14)

Further characterization of the gene expression profile at week 16 showed a similar trend of fold induction in genes encoding IFNβ, IFNγ, TNF, MCP-1, MIP-1α, IP-10, iNOS and IL-1β compared to controls (Figure 15). While transcription of *Il10* was not significantly changed, IFN-α and TGF-β were elevated (Figure 15). The role of TGF-β remains to be explored.

Thus, the BCG-*disA* OE treatment group the combination of higher expression of gene encoding Type I IFNs along with M1 and Th1 cytokines paired with modest expression of genes encoding regulatory cytokines correlates with a potent anti-tumor therapeutic effect in this rat model of bladder cancer. However, elucidation of the mechanism and further quantification of responses requires additional investigation.

5.3 Figures

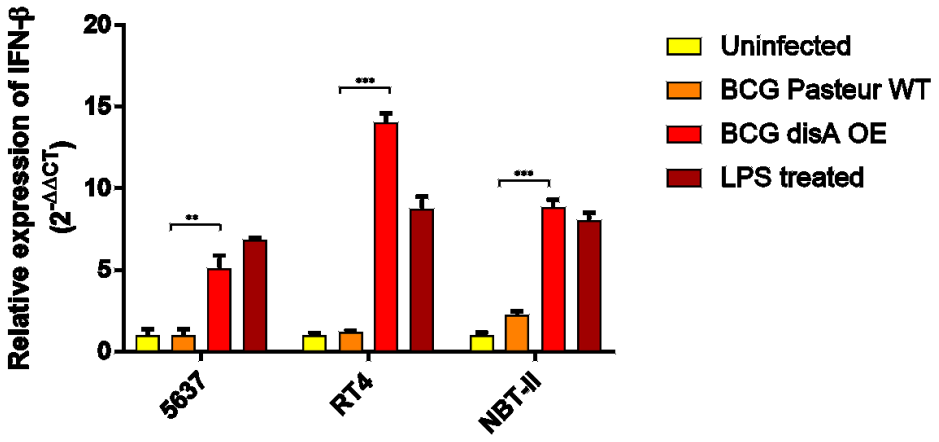

Figure 8. BCG overexpressing *disA* induces superior Type I IFN responses in all three Bca cell lines. Differential expression of interferon- β cytokines in bladder cancer cell lines challenged with wild-type and *disA* overexpression strains of *Mycobacterium bovis* BCG-Pasteur. BCa cells were challenged with wild-type and *disA* OE strains at an MOI of 1:20 for 5 hours to establish the infection. Uninfected bacteria were washed using ice-cold PBS and cells were subsequently incubated for another 3 hours. Expression levels of mRNA was measured using a SYBR green-based quantitative real-time PCR. Basal level of transcript (mRNA) in untreated macrophages was used for data normalization and hence to access relative expression. PPIB was used as an internal control in NBT-II and RNU6A was used as an endogenous control for RT4 and 5637 human cell lines. Data analysis was performed using $2^{-\Delta\Delta CT}$ method. The graphical points represent mean of three independent experiments ± standard error mean (SEM). Student's t test (*P < 0.05 **P < 0.01, ***P < 0.001). MOI (multiplicity of infection)

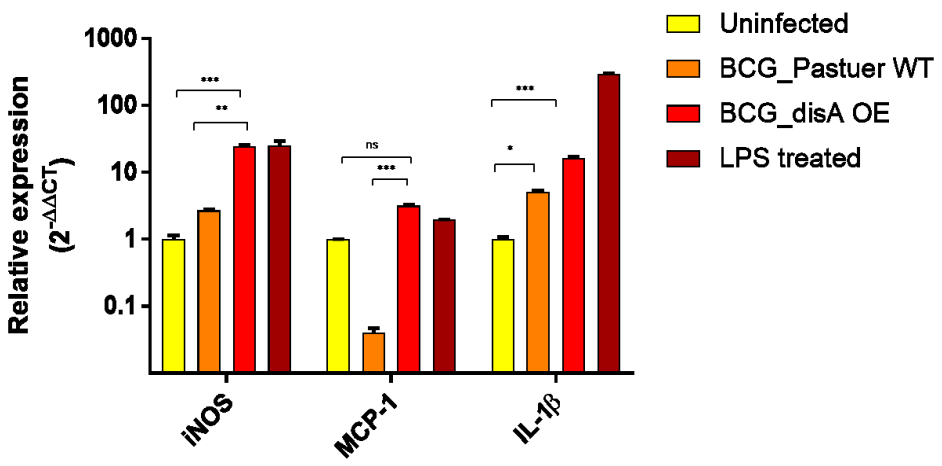

Figure 9. BCG overexpressing *disA* induces differential immune response in human bladder cancer cells (RT4). Differential gene expression in human RT4 bladder cancer cells challenged with wild-type, *disA* overexpression strains of Mycobacterium bovis BCG-Pasteur and *Mycobacterium bovis* BCG-Tice strain. Cells were challenged with wild-type and *disA* OE strains at an MOI of 1:20 for 5 hours to establish the infection. Uninfected bacteria were washed using ice-cold PBS and cells were subsequently incubated for another 6 hours. Expression levels of mRNA was measured using a SYBR green-based quantitative real-time PCR. Basal level of transcript (mRNA) in untreated macrophages was used for data normalization and hence to access relative expression. RNU6A was used as an internal control. Data analysis was performed using $2^{-\Delta\Delta CT}$ method. The graphical points represent mean of three independent experiments ± standard error mean (SEM). Student's t test (***P < 0.0005, **P<0.001). MOI (multiplicity of infection).

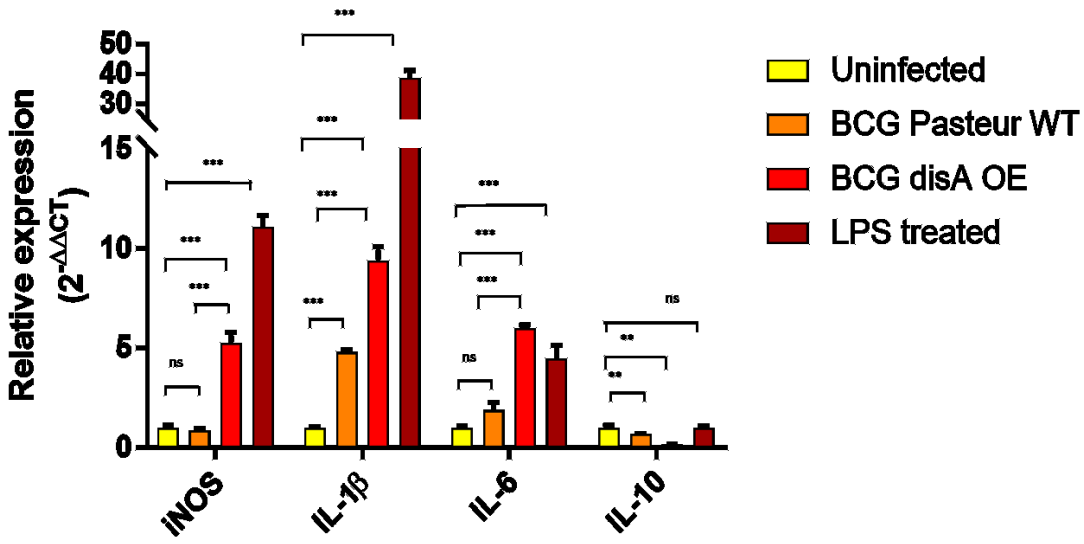

Figure 10. BCG overexpressing *disA* induces differential immune response in human bladder cancer cells (5637). Differential gene expression in human bladder cancer cells challenged with wild-type and *disA* overexpression strains of *Mycobacterium bovis* BCG-Pasteur. Cells were challenged with wild-type and *disA* OE strains at an MOI of 1:20 for 5 hours to establish the infection. Uninfected bacteria were washed using ice-cold PBS and cells were subsequently incubated for another 6 hours. Expression levels of mRNA was measured using a SYBR green-based quantitative real-time PCR. Basal level of transcript (mRNA) in untreated macrophages was used for data normalization and hence to access relative expression. RNU6A was used as an internal control. Data analysis was performed using $2^{-\Delta\Delta CT}$ method. The graphical points represent mean of three independent experiments ± standard error mean (SEM). Student's t test (***P < 0.0005, **P<0.001). MOI (multiplicity of infection).

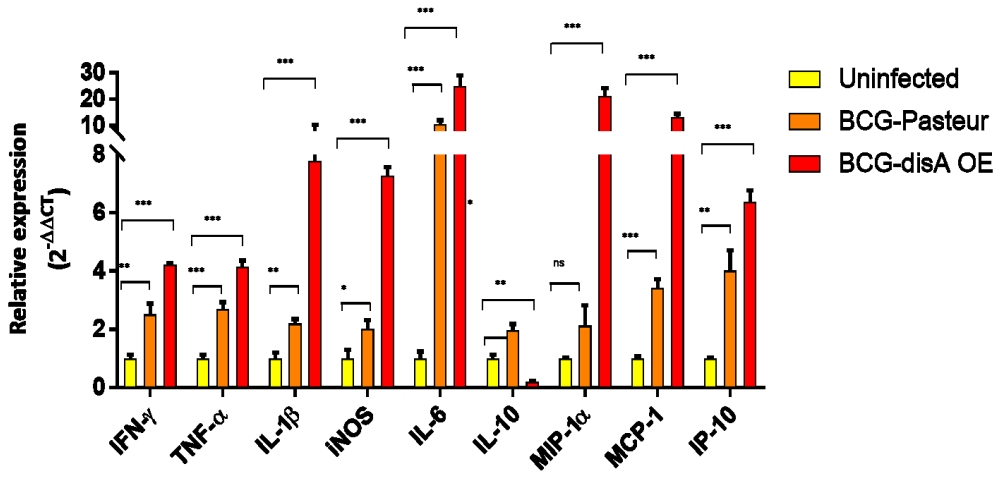

Figure 11 BCG overexpressing *disA* induces differential immune response in rat bladder cancer cells (NBT-II). Differential gene expression in rat bladder cancer cells challenged with wild-type and *disA* overexpression strains of *Mycobacterium bovis* BCG-Pasteur. Cells were challenged with wild-type and *disA* OE strains at an MOI of 1:20 for 5 hours to establish the infection. Uninfected bacteria were washed using ice-cold PBS and cells were subsequently incubated for another 6 hours. Expression levels of mRNA was measured using a SYBR green-based quantitative real-time PCR. Basal level of transcript (mRNA) in untreated macrophages was used for data normalization and hence to access relative expression. Peptidyl-prolyl cis-trans isomerase B (PPIB) was used as an internal control. Data analysis was performed using $2^{-\Delta\Delta CT}$ method. The graphical points represent mean of three independent experiments ± standard error mean (SEM). Student's T test (***P < 0.0005, **P<0.001). MOI (multiplicity of infection).

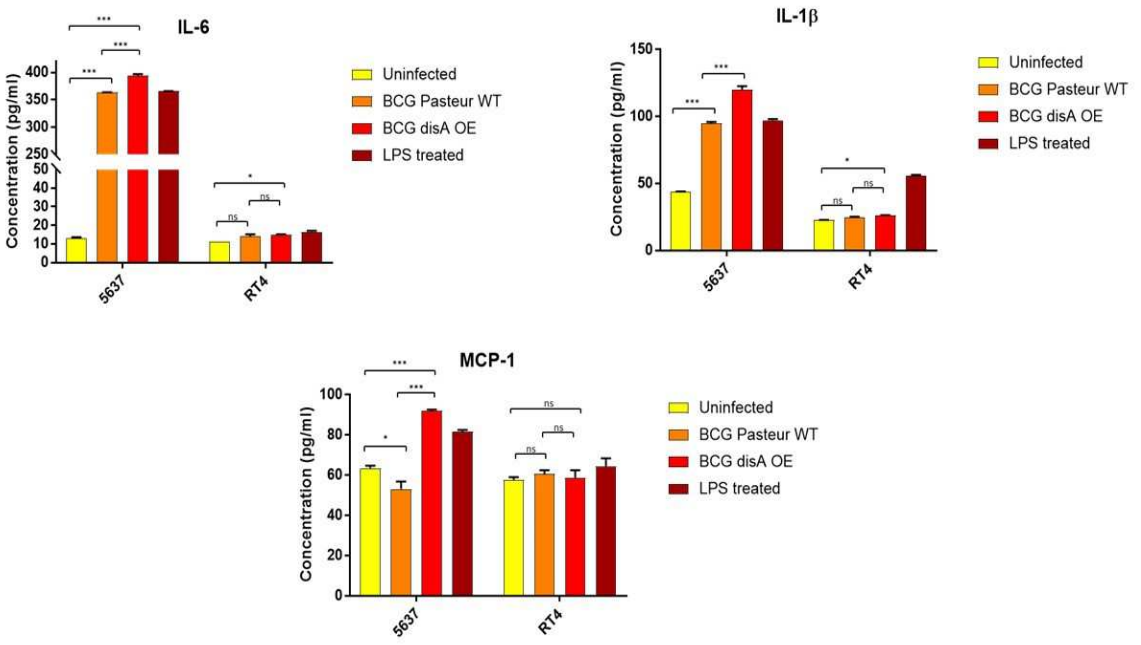

Figure 12. Increased pro-inflammatory cytokines in response to *disA* overexpression in Bca cell lines (ELISA). Differential expression of IL-6 **(a)**, MCP-1 **(b)** and IL-1β **(c)** in two human bladder cancer cell lines (5637, RT4) challenged with wild-type and *disA* overexpression strains of *Mycobacterium bovis* BCG-Pasteur. Cells were challenged with wild-type and *disA* OE strains at an MOI of 1:20 for 5 hours to establish the infection. Uninfected bacteria were washed using ice-cold PBS and cells were subsequently incubated for another 24 hours. Culture supernatants were assayed by ELISA for different cytokines. The graphical points represent mean of three independent experiments ± standard error mean (SEM). Student's t test (*P < 0.05 **P < 0.01, ***P < 0.001). MOI (multiplicity of infection)

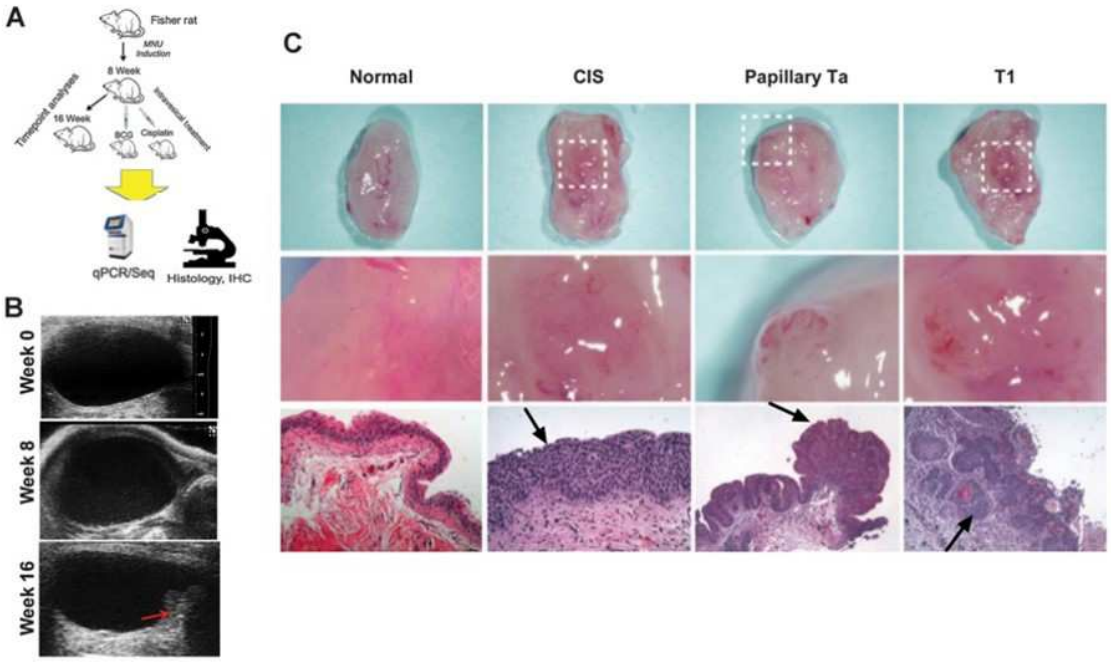

Figure 13. The MNU Model of Bladder Cancer Displays a histopathologic phenotype similar to that of human urothelial cancer by Bivalaqua laboratory (A) Fischer rats are given 4 biweekly instillations of MNU. For time point analyses, bladders were harvested at 8 and 16 weeks. For treatment studies, animals were given 6 weekly doses of BCG and BCG-*disA* OE from week 8-14. Bladders were then harvested at week 16 for RT-PCR and histology (B) By 16 weeks MNU treated bladders had evidence of increased thickening and papillary tumors on ultrasound imaging, and (C) histologic examination demonstrated a combination of papillary Ta, CIS, and T1 cancer (arrows point to relevant stage). All experiments were performed 4 times.

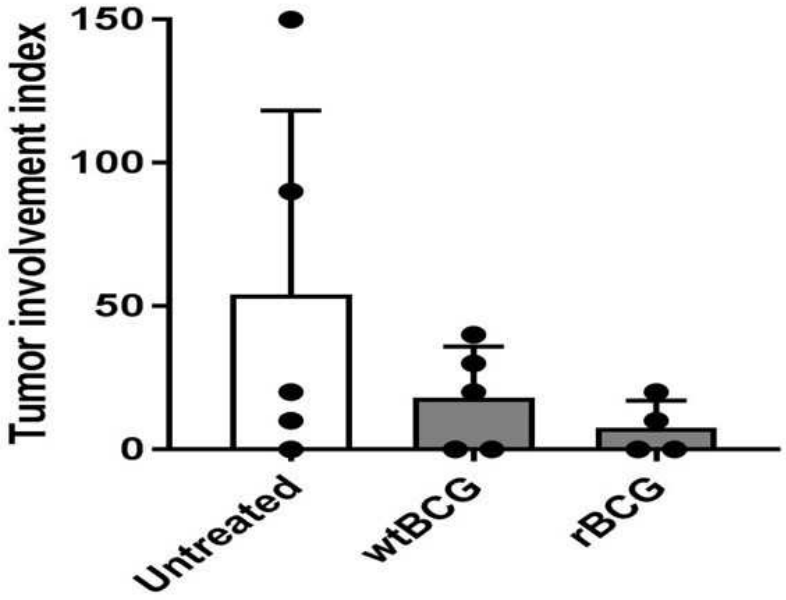

Figure 14. Tumor involvement index of tumor-bearing rats untreated or treated with WT BCG or BCG-*disA* OE. All specimens (n =5) at week 16 had evidence of CIS, papillary Ta, or high- grade T1 urothelial carcinoma by analogy with the 2004 World Health Organization/International Society of Urological Pathology classification system. Tumor staging will be performed by a certified genitourinary pathologist blinded to the treatments.

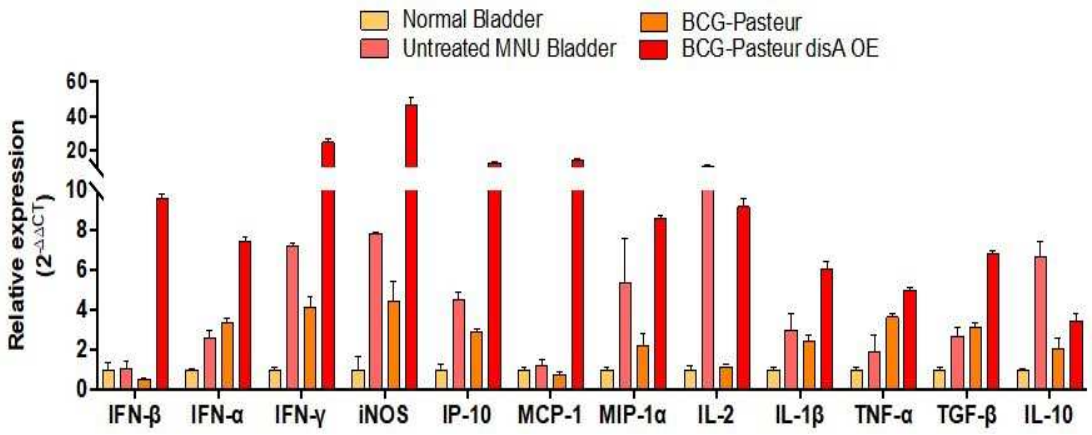

Figure 15. Immune profiling of MNU-induced Fisher rat urinary bladder tumors in response to intravesical therapy using different strains of BCG. Differential gene expression in Rat bladder tumor cells after therapy with wild-type and *disA* overexpression strains of *Mycobacterium bovis* BCG-Pasteur. Expression levels of mRNA was measured using a SYBR green-based quantitative real-time PCR. Basal level of transcript (mRNA) in untreated macrophages was used for data normalization and hence to access relative expression. Peptidyl-prolyl cis-trans isomerase B (PPIB) was used as an internal control. The graphical points represent mean of three independent experiments ± standard error mean (SEM). Student's t test (*P < 0.05 **P < 0.01, ***P < 0.001). MOI (multiplicity of infection).

Future Work

Overall our *in vitro* and *in vivo* experiments suggest a favorable immune response that characteristically matches with the successful BCG immunotherapy. We used different cell types ranging from macrophages (human and mouse), bladder cancer cells (human and rat) and a rodent model if bladder cancer to validate our proof of concept that BCG strains overexpressing STING agonists can be a better therapeutic option for bladder cancer immunotherapy. Key questions that remain to be answered include whether this recombinant strain of BCG can induce a long-lasting memory response, the optimal levels of c-di-AMP from the recombinant bacteria, whether there are tolerogenic effects and whether the recombinant bacterial strains are secreting c-di-AMP inside infected cells independent of the ESX-1 pathway.

Appendix

IFNβ: Interferon beta

IFNα: Interferon alpha

Type I IFNs: Interferon α and Interferon β

IFNα2b: Interferon alfa 2b

IFNα2a: Interferon alpha 2a

IFNγ: Interferon gamma

IL-1β: Interleukin 1 beta

TNF: Tumor necrosis factor alpha

IL-6: Interleukin 6

Il-2: Interleukin 2

IL-12: Interleukin 12

IL-18: Interleukin 18

IP-10: Interferon gamma-induced protein 10

MCP-1: Monocyte chemoattractant protein 1

IL-10: Interleukin 10

MIP-1α: Macrophage Inflammatory Protein 1 alpha

TGF-β: transforming growth factor beta

Arg-1: Arginase 1

CCL2: C-C motif chemokine ligand 2 (also MCP-1)

MDC: Macrophage-derived chemokine

MIG: Monokine induced by gamma interferon

STING: Stimulator of interferon genes

c-di-AMP: Cyclic diadenylate monophosphate

TBK1: Serine/threonine-protein kinase

CDN: Cyclic di nucleotides

NF-κb: Nuclear factor kappa B

ISG: interferon-stimulated gene

PD-1: Programmed cell death protein 1

CSP: *cytosolic* surveillance pathway

c-di-IMP: Cyclic di-inosine monophosphate

cGAMP: Cyclic guanosine monophosphate–adenosine monophosphate

cGAS: Cyclic GMP-AMP synthase

GTP: Guanosine triphosphate

DAMP: danger-associated molecular patterns

IRF3: interferon regulatory factor-3

IRF: Interferon regulatory factor

iNOS: Inducible nitric oxide synthase

PAMP: Pathogen-associated molecular pattern

PRRs: Pattern recognition receptors

TLR2: Toll-like receptor 2

TLR9: Toll-like receptor 9

NOD2: Nucleotide-binding oligomerization domain-containing protein 2

NLRP3: NACHT, LRR and PYD domains-containing protein 3

AIM-2: Interferon-inducible protein AIM2, or absent in melanoma 2

dsRNA: double stranded RNA

c-AMP: Cyclic adenosine monophosphate

c-di-GMP: cyclic dimeric guanosine monophosphate

cGMP-AMP: Cyclic guanosine monophosphate–adenosine monophosphate

RD-1: Region of difference 1 gene locus

STAT6: Signal Transducers and Activators of Transcription

MyD88: Myeloid differentiation primary response 88

Sox2: sex determining region Y

DAI: DNA-dependent activator of interferon-regulatory factors

IFI16: interferon-inducible myeloid differentiation transcriptional activator gene

DDX41: DEAD box helicase 41

Iκκ: IκB kinase

ERAdP: endoplasmic reticulum (ER) membrane adaptor

Ikb: nuclear factor of kappa light polypeptide gene enhancer

ESAT: early secretory antigen target

GM-CSF: Granulocyte-macrophage colony-stimulating factor

NO: nitric oxide

EGFR: epidermal growth factor receptor

iNOS: nitric oxide synthase

DMXAA: 5,6-dimethylxanthenone-4-acetic acid

mSTING: Mouse Stimulator of interferon genes

hSTING: human Stimulator of interferon genes

ADU-S100: Aduro Sting agonist

MK-1454: Merck's CDN Sting agonist

ATP: Adenosine triphosphate

ADP: adenosine diphosphate

MAVS: Mitochondrial antiviral-signaling protein

Poly I:C: polyinosinic-polycytidylic acid

disA: DNA integrity scanning protein gene

PDE: phosphodiesterase

MUC-1: Mucin 1, cell surface associated

disA/dacA: DNA integrity scanning protein

sigH: RNA polymerase sigH factor

PPIB: Peptidyl-prolyl cis-trans isomerase B

RNU44: Small Nucleolar RNA

BCG: Bacillus Calmette–Guérin

OE: Overexpressor

BCG-*disA* OE: Recombinant BCG Overexpressing disA gene

M.tb-CDC 1551: Mycobacterium tuberculosis CDC1551 strain

M. bovis: Mycobacterium bovis

BCG-hIL2-MUC1: BCG expressing IL-2 and MUC1

rBCG: Recombinant BCG

HIV: Human immunodeficiency virus

S. aureus: Staphylococcus aureus

L. monocytogenes: Listeria monocytogenes

B. subtilis: Bacillus subtilis

MBT-2: FANFT-induced bladder tumour in C3H/He mouse

PEC: Bacille Calmette–Guérin (BCG)-induced peritoneal exudate cells of mice

T24: Human hypodiploidy to hypopentaploidy female epithelial transitional cell carcinoma cell line

J82: human bladder carcinoma cell line

5637: human bladder polyploidy male cancer urinary bladder grade II carcinoma cell

UMUC-3: Human hypertriploid male epithelial transitional cell carcinoma cell line

TCCSUP: Human hypotetraploid female grade IV transitional cell carcinoma cell line

PBMC: peripheral blood mononuclear cell

MB49: Cells from C57BL mouse bladder epithelial cells transformed with chemical carcinogen 7, 12-dimethylbenz[a]anthracene (DMBA)

J774A.1: *Mus musculus* BALB/cN *derived macrophage cell line*

THP-1: monocyte lineage cell line from Human acute monocytic leukemia

RT4: Human tetraploid male derived transitional cell papilloma cell line

NBT-II: Nara Bladder Tumor No. 2 cell line induced by N-butyl-N-(4-hydroxybutyl) nitrosamine in rat cells

RAW 264.7: *Mus musculus* derived Macrophage/ monocytic cell line derived from Abelson murine leukemia virus-induced tumor

CT26: N-nitroso-N-methylurethane-(NNMU) induced, undifferentiated colon carcinoma cell line

DCs: Dendritic cells

NK cells: Natural killer cells

CD4: Cluster of differentiation 4

CD8: Cluster of differentiation 8

BMDM: Bone-marrow-derived macrophage

Th1: Type 1 T helper

Th2: T helper 2 cells.

Th17: T helper 17 cell

Tregs: Regulatory T cell

CD: Cluster of differentiation

M1: Classically activated macrophages

M2: Alternatively, activated macrophages

NMIBC: Non-muscle-invasive bladder cancer

MNU: N-Nitroso-N-methylurea

CIS: Carcinoma in situ

TUR: Transurethral resection

PEGylated: Amalgamation of polyethylene glycol

C22b2b: Immunocytokine comprising tetrameric. IFNα2b coupled to hL243

hL243: humanized monoclonal antibody that is specific for HLA-DR

FANFT: N-[4-(5-Nitro-2-furyl)-2-thiazolyl]formamide

BBN: N-butyl-N-(4-hydroxybutyl)-nitrosamine

TME: Tumor microenvironment

Ta: non-invasive papillary carcinoma, which is grown toward the center of the bladder and has not spread to distant sites nor lymph nodes

T1: The tumor has spread to the lamina propria) but not bladder wall muscle

AIDS: acquired immune deficiency syndrome

TB: Tuberculosis

SCID: Severe combined immunodeficiency

PCR: Polymerase chain reaction

ELISA: Enzyme-linked immunosorbent assay

OADC: Oleic Albumin Dextrose Catalase

CTAB: Cetyl trimethylammonium bromide

qPCR: real-time polymerase chain reaction

SYBR: SYBR Green I

RPMI: Roswell Park Memorial Institute media

DMSO: Dimethyl sulfoxide

FBS: Fetal Bovine Serum L929- Mus musculus L cell, L-929, derivative of Strain L

PBS: Phosphate-buffered saline

SEAP: Secreted alkaline phosphatase

MOI: Multiplicity of infection

DMEM: Dulbecco's Modified Eagle Medium

7H9: Middlebrook 7H9 broth

7H11: Middlebrook 7H11 agar

SEM: Standard error of the mean

Mab: Monoclonal antibody

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Curriculum Vitae| Monali Praharaj

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EDUCATION

Masters in Science, Molecular Microbiology and Immunology
Ongoing
Johns Hopkins Bloomberg School of Public Health
August 2016 -2018

Bachelor of Technology, Biotechnology
Gold Medalist
Dr. DY Patil University Navi Mumbai, India
July 2010- 2014

RESEARCH EXPERIENCE

William Bishai Lab, TB research Centre, Johns Hopkins School of Medicine
January 2017- Present
Master's Thesis Candidate, Graduate fellow

- Generation of recombinant BCG strains with improved antigenic repertoire, as an immunotherapeutic therapy against urinary bladder cancer and testing therapeutic efficacy using *in vivo* Fisher Rat model of Bladder Cancer (in collaboration with Dr Trinity Bivalacqua, James Buchanan Brady Urologic Institute)
- Generation of recombinant *M. tuberculosis* strains with increased secretory antigens to investigate the role of secreted mimic proteins for their immune subversive potential using in vitro and *in vivo* mouse model of tuberculosis.
- Used gene manipulation in mycobacteria, gene expression analyses (TaqMan based qPCR), Immunoassays (ELISA, Flow cytometry, Western blots), *In vivo* (mice handling-immunizations, establishment of TB infection using aerosol method, preparation of single cell for immunoassays), *In vitro*- primary macrophage and dendritic cell culture using mouse bone marrow, infection assays, and handling different cancer cell lines
- Certified to work in bsl3 and absl3.

Arturo Casadevall Lab, Johns Hopkins School of Public Health
November 2016- January 2017
Master's Researcher

- Studied the role of moonlighting properties of cryptococcal urease in macrophage and *Cryptococcus neoformans* interaction
- Used time lapse (Zeiss Axiovert 200M microscope), fluorescent ratio imaging (intracellular macrophage pH measurements), optimized enzyme kinetic assays, growth kinetics of mutants to study the secondary functions of *Cryptococcal* urease in murine derived macrophages.

MGM OMICS Research Centre, Navi Mumbai, India
November 2015- June 2016
Junior Research Assistant

- Identification of blood based biomarkers (LipA, lipases, non-specific lipids) for Malaria and devising rapid zymogram diagnostic assays.
- Efficiently handled patient samples and developed zymographic technique on the basis of $sPLA_2$ protein to differentiate the infecting strain of Malaria.

Cell and Tissue Engineering lab, Indian Institute of Technology, Mumbai, India
March 2015- May 2015
Project Technical Assistant
- Sub cloned gene of interest-chicken ovalbumin (OVA) antigen specific T cell receptor in lentiviral vector plasmid with EF1 alpha promoter and RFP as a selection marker. Further, optimized transfection conditions with branched PEI of pGipz plasmid into HEK293FT cells

Genomics and Systems Biology lab, Indian Institute of Technology, Mumbai, India
July 2014- January 2015
Junior Research Fellow
- Researched the molecular mechanisms behind acquired drug resistance in Mycobacterium smegmatis by mRNA analysis and biochemical assays. Identified the target mutations responsible for multi drug resistance by gene sequencing
- Used Real time PCR based assays, Electron microscopy (Transmission and Scanning), Molecular Biology techniques, Fluorescence and Confocal microscopy.

Genomics and Systems Biology lab, Indian Institute of Technology, Mumbai, India
Dec 2013- June 2014
Intern, Graduate thesis
- Studied the growth, morphology and transport patterns in drug resistant and multi- drug resistant (MDR) mutants of Mycobacterium smegmatis.

PUBLICATIONS

Jain-Dey R, Dey B, Singh AK, **Praharaj M**, Srikrishna G, Bishai W. A novel rBCG overexpressing c-di-AMP in more potent than BCG in the guinea pig TB model. 2017 in preparation

Singh US, **Praharaj M**, Sharma C, Das A (2016) Paradigm Shift in Transmission of Vector Borne Diseases. J Emerg Infect Dis 1:116. doi: 10.4172/2472-4998.1000116

GRANT & MANUSCRIPT EDITING

- Wei Zhang, Shichun Lun, Shu-Huan Wang,Fan Yang, Jie Tang, Hendra Gunosewoyo, William R. Bishai, and Li-Fang Yu. Identification of Novel Coumestan Derivatives as Pks13 Inhibitors against Mycobacterium tuberculosis. 2017 in preparation.
- The Maryland Innovation Initiative (MII) grant: A potent rBCG as a therapeutic intervention for non-muscle invasive bladder cancer. Principal Investigator: William R Bishai, Trinity Bivalacqua
- Bioemergent Fellowship Grant: A Potent rBCG as a therapeutic intervention for non-muscle invasive bladder cancer. Monali Praharaj, ScM candidate, 2018 Mentors: William Bishai, MD PhD (Medicine), Trinity Bivalaqua, MD, PhD (Urology)

AWARDS

2017 **Receiving graduate level fellowship, Master's thesis project,** William Bishai Laboratory,

Tuberculosis Research Centre, Division of Infectious Disease, Johns Hopkins School of Medicine

2016 **Selected for National Level Junior Research Fellowship**, Council for Scientific and Industrial Research, India

2016 **Qualified National Level Joint Graduate Entrance Examination** for Biology and Interdisciplinary Life sciences, National Centre for Biological Sciences, India

2014 **Academic Excellence Scholarship** and Certificate for four academic semesters B.tech Biotechnology in D.Y. Patil Department of Biotechnology and Bioinformatics

MEMBERSHIP & EXTRACURRICULAR ACTIVITIES

Member, American Society for Microbiology
Johns Hopkins School of Public Health, Baltimore

Elected Forum Student Coordinator, Molecular Microbiology and Immunology Department
Johns Hopkins School of Public Health

Communications Intern, JHIEPGO *(Nov 16'- Jan 17')*
Global Engagement and Communications Department

Facilitator, Fundraising and NGO visits (*Dec 11-12')*
AIESEC Navi Mumbai

Selected Delegate, 2nd UN Young Changemakers conclave *(April 12')*
US Consulate, Mumbai, India

CONFERENCES & WORKSHOPS

ASM Bio threats: Research, Response, and Policy 2017 American Society of Microbiology

Tropical Medicine Dinner Club Meetings Johns Hopkins School of Public Health
Dengue Vaccines Dinner Club (Oct 5, 2016)
Zika: Should we be worried? (November 9, 2016)

Advancing TB Research (Symposium- April 2016) P.D. Hinduja Hospital & Medical Research Centre
In association with The Division of AIDS (NIAID & NIH)

National Symposium on Tuberculosis (Global STOP TB Initiative) MGM Hospital Navi Mumbai, India